A3 CONT.

co-administering to said mammal with said compound another chemotherapeutic agent effective for the treatment of the malignant disease or condition of the mammal where the composition in combination with the other chemotherapeutic agent shows synergistic effect.

REMARKS

The Grounds for Objections to Claims Relating to Form Have Been Obviated by the Present Amendment

Commas and a semi-colon have been inserted in the places suggested by the Examiner, thereby obviating these grounds of objections.

The Objection that Certain Claims are Substantial Duplicates of Other Claims is Respectfully Traversed

Claims 3 and 4 were objected to as being substantial duplicates of Claim 2. Similar objections were made to Claim 7 and 8 and to 11 and 12. These objections are respectfully traversed. The reason is as follows. Claim 2 calls for a composition that is effective for treatment of a malignant disease or condition. Claim 3 calls for a composition that is suitable for treatment of breast cancer, and Claim 4 calls for a composition that is suitable for treatment of leukemia. Clearly, a malignant disease or condition is a *broader* category than breast cancer or leukemia. Therefore, Claim 2 is generic in relation to Claims 3 and 4, which can be considered species claims. Similar consideration apply to Claims 7 and 8 which are species claims relative to Claim 6, and to Claims 11 and 12 which are species claims relative to Claim 10.

The above noted argument or logic applies even more forcefully to method of treatment claims 17 and 18, which are species claims relative to Claim 16, to Claims 22 and 23 (species of Claim 21) and to Claims 28 and 29 which are species of Claim 27. The kind of disease or condition which is treated in a claimed method of treatment claim is clearly a proper limitation of the claim. Using Claim 17 as an example, treatment of breast cancer or leukemia is clearly a narrower concept than the treatment set forth in the parent Claim 14 and also in the

intervening Claims 15 and 16 which is for malignant disease or condition. For these reasons the objection on the grounds that the claims are substantial duplicates of other claims is respectfully requested to be reconsidered and withdrawn.

The Grounds for Rejecting Certain Claims for Indefiniteness Have Been Obviated in-part, and Are Respectfully Traversed in-part

The definition of the variable X has been inadvertently omitted from the two independent Claims of the application. This inadvertent error has now been rectified by amendment of these two claims. **X** is now defined as "O or S". This definition does not introduce new matter because the specification amply supports this definition, see for example Page 7 lines 12 and 13. In order to conform the Summary of the Invention to Claims1 and 14, the Summary was also amended to include this definition of **X**.

The ground for the rejection that the claims are indefinite because dosing ranges are not set forth in Claims 1 and 14 is respectfully traversed. The specification teaches in essence that the daily dose of the compounds of **Formula 1** as well as the daily dose of the other chemotherapeutic agent of the combination therapy will vary based on the nature and severity of the condition to be treated, and can be arrived at "through routine experimentation, which is customary in the science of the chemotherapy of malignancies." See pages 16 through 18 of the specification, and particularly page 17, lines 18 – 19. It is respectfully submitted that exactly as the specification teaches, one having ordinary skill in the art would be able to arrive at the necessary effective doses by routine experimentation and not by undue experimentation. Therefore, setting forth the ranges in these two main claims is not necessary in order to comply with the requirements of 35 U. S. C. Section 112 second paragraph. It is also noted that the specification nevertheless teaches a likely dose range of 50 mg to 500 mg per day for compounds of **Formula 1**, and 1 to 9 million international units per day when the other chemotherapeutic agent of the combination is interferon.

The Rejection for Lack of Enablement Pursuant to 35 U. S. C. Section 112, First Paragraph Is Respectfully Traversed

The rejection on the grounds that the specification enables only inhibition of growth of certain i*n vitro* cell lines (as specified in the Office Action) and not treatment of mammals afflicted with a condition of tumors, is respectfully submitted to be in error, and its reconsideration and withdrawal are respectfully requested. The reasons are as follows.

First, it appears that the rejection stated in the Office Action for alleged lack of enablement pursuant to 35 U.S.C. Section 112, First Paragraph is in fact a rejection for lack of utility pursuant to 35 U.S.C. Section 101. As it is stated in the Manual of Patent Examining Procedure §2107.01

> "Courts have repeatedly found that the mere identification of a pharmacological activity of a compound that is relevant to an asserted pharmacological use provides an 'immediate benefit to the public' and thus satisfies the utility requirement. As the Court of Customs and Patent Appeals held in *Nelson v. Bowler*:
>
> > 'Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. It is inherently faster and easier to combat illnesses and alleviate symptoms when the medical profession is armed with an arsenal of chemicals having known pharmacological activities. Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many compounds as possible, we conclude that adequate proof of any such activity constitutes a showing of practical utility."

The Manual of Patent Examining Procedure §2107.01 also addresses the question whether data from *in vitro* testing satisfy the utility requirement, and states:

"Similarly, courts have found utility for therapeutic inventions despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition. The Federal Circuit in *Cross v. Izuka*, 753 F.2d 1040, 1051, 244 USPQ 739, 747-748 (Fed. Cir. 1985), commented on the significance of data from *in vitro* testing that showed pharmacological activity:

> 'We perceive no insurmountable difficulty, under appropriate circumstances, in finding that the first link in the screening chain, *in vitro* testing, may establish a practical utility for the compound in question. Successful *in vitro* testing will marshal resources and direct the expenditure of effort to further *in vivo* testing of the most potent compounds, thereby providing an immediate benefit to the public, analogous to the benefit provided by the showing of an *in vivo* utility.'"

The Manual of Patent Examining Procedure §2107.01 states further that "The Federal Circuit has reiterated that therapeutic utility sufficient under the patent laws is not to be confused with the requirements of the FDA with regard to safety and efficacy of drugs to be marketed in the United States.

> 'FDA approval, however is not a prerequisite for finding a compound useful within the meaning of the patent laws. [*Scott v. Finney* 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994)] Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new

inventions., thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas, such as the treatment of cancer.'

In re Brana, 51 F3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995) Accordingly Office personnel should not construe 35 U.S.C. 101 under the logic of 'practical' utility or otherwise, to require that an applicant demonstrate that the therapeutic agent based on a claimed invention is safe or fully effective drug for humans."

The above noted instructions from the M.P.E.P. and holdings from the Courts overseeing the patent laws, are fully applicable in the instant case. The instant specification indisputably describes modes of administration and even likely doses of the compounds of the invention in combination with other chemotherapeutic agents (see pages 16 through 18). It is also noted that the specification incorporates by reference several U.S. patents which disclose the synthetic procedures for the preparation of all compounds within the scope of the claims.

The *in vitro* testing data clearly show positive, in fact synergistic results, and thus demonstrates utility and enables the invention within the requirements of the law. Moreover, the pertinent art itself recognizes *in vitro testing* as an indication that compounds or compositions capable of inhibiting the growth of tumor cell lines *in vitro* are likely candidates for clinical trials for treatment of humans. A sample of scientific publications proving this recognition in the art of *in vitro* cell line testing is enclosed here with the most pertinent parts highlighted in the publications.

For example, in the publication by *Hassan et. al.* in J. Cancer Res. Clin. Oncol. (1991) 117:227-231 the authors state regarding a composition that was found synergistically active with another agent in an *in vitro* cell line, "[T]hus, the present combinations could provide shorter and less toxic courses of treatment in elderly myeloid leukaemic patients."

In European Journal of Pharmacology 424 (2001) 1 - 11 the authors (*Vondracek et al.*) describe *in vitro* synergistic results in experiments with indomethacin in combination with another agent, and state that "our data raise the possibility that indomethacin could potentially be used to improve the treatment of human myeloid leukaemia."

In Cancer Chemother. Pharmacol. (1966) 37: 22 – 228 the authors (*Ting-Chao et al.*) describe synergistic results of taxol or taxotere with edetrexate in *in vitro* cell line experiments, and state, that "[T]hese results show potent schedule-dependent synergism of the combinations of TXL or TXT with EDX, and should form a rationale for designing clinical protocols utilizing these agents particularly for the treatment of breast cancer patients."

In Eur. Surg. Res. 2001; 33:232-236 the drug 5-fluorouracil was found to have synergistic effect in an *in vitro* cell line with another agent, and the authors (*Tsunoda et al.*) stated "[A]dministering 5-FU after preincubation with CGS16949A significantly increased the combined cytotoxic efficacy, suggesting that clinical therapy using this combined therapy may be more efficient."

In the publication Anticancer Research 21:3209- 3214 (2001) *Schultz et al.* describe *in vitro* cell culture results of the combination doxorubicin with the drug Alimta™, and based on the *in* vitro data state, that "to obtain maximal antitumor activity, Alimta should precede doxorubicin when the drugs are given in combination therapy protocols."

In light of all of the foregoing, the rejection for lack of enablement, or lack of utility is in error and should not be maintained. In the event the Examiner is of the view that the data presented in the specification are incorrect, or if the Examiner is aware of facts which would negate the utility of the method claimed in the application, he is respectfully requested to set forth those facts in an affidavit or signed declaration form.

Rejection of the Claims for Obviousness Is in Error and Should Not Be Maintained

The claims were rejected as defining allegedly obvious subject matter in view of a combination of references. This ground of the rejection is respectfully submitted to be in error and is respectfully traversed.

The principal references in support of the rejection are *Chandraratna* U. S. Patent No. 5,045,551, *Marth et al.* JNCL. Vol. 77 No. 6 pp 1197 – 1202, and *Nara* Leukemia and Lymphoma Vo. 10, pp 201 207. It is true that the cited *Chandraratna* patents describe compounds of identical or nearly identical structures with the compounds of **Formula 1** in the claimed method. However, none of the cited *Chandraratna* patents disclose a composition or method of combination therapy which results in *synergistic* effects. The presently amended claims now clearly recite that the claimed combination or method of using the claimed combination results in synergistic effects.

The *Marth et al.* reference describes a combination of retinoic acid with interferon that results in a synergistic effect in inhibiting the growth of certain malignant cell lines. The Office Action asserts that it would have been obvious to one of ordinary skill in the art to substitute the instantly claimed compounds of **Formula 1** for retinoic acid in the composition of *Marth et al.*. This conclusion, however, is in error because of the vast structural difference between retinoic acid and the compounds of **Formula 1**. Whereas retinoic acid is a cyclohexene derivative attached to a chain of conjugated double bonds, the compounds of **Formula 1** are chroman or thichroman derivatives including a triple bond and a phenyl or heteroaryl group. More preferred compounds of the claimed composition and method include a thiochroman moiety attached with a triple bond to a pyridine moiety (Claims 5 – 13, and 19 – 30.) Given this vast structural difference, one of ordinary skill in the art would have had no reason, much less an obvious reason, to expect that the compositions of **Formula 1** would provide synergistic results when used in combination with other chemotherapeutic agents.

The *Nara* reference when combined with any of the cited *Chandraratna* patents gives rise to even less ground to support a holding of obviousness. None of the factors or compositions described in the *Nara* reference are even remotely related to the compounds of **Formula 1**. For this reason a person of ordinary skill in the art would have had absolutely no reason, much less an obvious reason, to expect synergistic effect for the combination of the compounds of **Formula 1** with other chemotherapeutic agents. For all of the foregoing reasons the rejection of the claims for obviousness should be reconsidered and withdrawn.

In light of the foregoing all claims are sincerely believed to be in *prima facie* allowable condition. In the event the Examiner is of the opinion that a telephone conference with the undersigned attorney would materially facilitate the final disposition of this case, he is respectfully requested to telephone the undersigned attorney at the below listed telephone number.

Although a separate change of address notice (form PTO-SB/122 (10-01) is being sent to the Patent Office for this case, the Examiner is respectfully requested to take note of the undersigned attorney's new address and telephone number.

Respectfully submitted,

Gabor L. Szekeres

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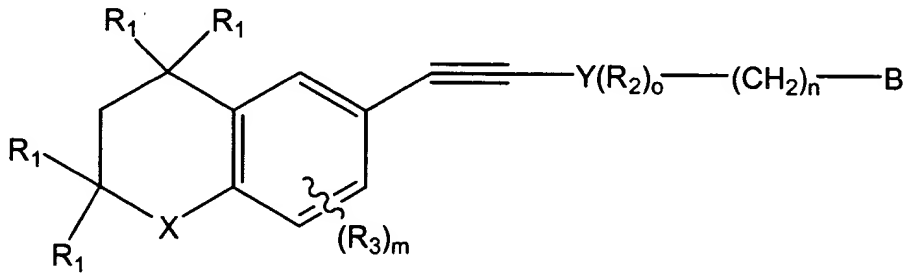
VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claim 1 (amended)

A pharmaceutical composition for the treatment of a malignant disease or condition in a mammal, the composition comprising a pharmaceutically acceptable excipient and a therapeutically effective dose of a compound of the formula



where X is S or O;

R_1 is, independently, H or lower alkyl of 1 to 6 carbons;

R_2 and R_3 are, independently, H, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, alkoxy of 1 to 6 carbons, or fluoroalkoxy of 1 to 6 carbons;

m is an integer 0 to 3;

o is an integer 0 to 4;

n is 0-5;

Y is phenyl, naphthyl, or a heteroaryl group selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl; oxazolyl, thiazolyl, or imidazolyl[,]; and

B is COOH, a pharmaceutically acceptable salt thereof, $CONR_6R_7$ or $COOR_8$ where R_6 and R_7, independently, are hydrogen or an alkyl group of 1 to 6 carbons and R_8 is alkyl of 1 to 6 carbons,

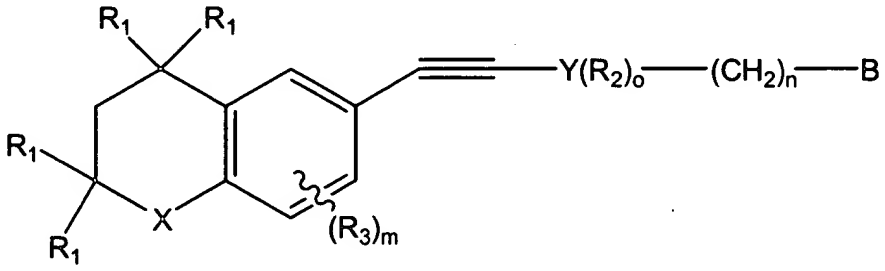
said composition being adapted to be used in combination with another chemotherapeutic agent effective for the treatment of the malignant disease or

condition of the mammal[.] where the composition in combination with the other chemotherapeutic agent shows synergistic effect.

Claim 14 (amended)

A method of treating a malignant disease or condition in a mammal in need of such treatment, the method comprising the steps of:

administering to said mammal a pharmaceutical composition comprising a pharmaceutically acceptable excipient and a therapeutically effective dose of a compound of the formula



where X is S or O;

R_1 is, independently, H or lower alkyl of 1 to 6 carbons;

R_2 and R_3 are, independently, H, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, alkoxy of 1 to 6 carbons, or fluoroalkoxy of 1 to 6 carbons;

m is an integer 0 to 3;

o is an integer 0 to 4;

n is 0-5;

Y is phenyl, naphthyl, or a heteroaryl group selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl; oxazolyl, thiazolyl, or imidazolyl;

B is COOH, a pharmaceutically acceptable salt thereof, $CONR_6R_7$ or $COOR_8$ where R_6 and R_7, independently, are hydrogen or an alkyl group of 1 to 6 carbons and R_8 is alkyl of 1 to 6 carbons, and

co-administering to said mammal with said compound another chemotherapeutic agent effective for the treatment of the malignant disease or condition of the mammal[.] where the composition in combination with the other chemotherapeutic agent shows synergistic effect.

J Cancer Res Clin Oncol (1991) 117:227–231
0171521691000042Y

Cancer Research
Clinical Oncology

Synergistic interactions between differentiation-inducing agents in inhibiting the proliferation of HL-60 human myeloid leukaemia cells in clonogenic micro assays

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Summary. All-*trans*-retinoic acid, hexamethylene bisacetamide and 5-azacytidine are inducers of granulocytic differentiation of HL-60 human myeloid leukaemic cells, which eventually leads to inhibition of cell proliferation. The effect of graded concentrations of all-*trans*-retinoic acid (RA) (1 n*M*–1 μ*M*), hexamethylene bisacetamide (HMBA) (0.5–4 m*M*) and/or 5-azacytidine (5azaC) (1 n*M*–1 m*M*), alone and in combination with each other on colony formation and growth of HL-60 cells was studied in agar capillary clonogenic micro assays in order to identify new potential therapeutic regimens for elderly patients with acute myeloid leukaemia. ED_{90} concentrations, inducing 90% inhibition of colony formation for RA, HMBA and 5azaC, were 128 n*M*, 2.7 m*M* and 40 μ*M*, respectively. The drug interactions between these differentiating agents were analysed by Berenbaum's general algebraic solution. The combinations: RA + HMBA, 5azaC + HMBA and RA + 5azaC were significantly synergistic in inhibiting HL-60 colony formation. Their interaction indices were 0.62, 0.83, and 0.97, respectively, at a specific effect level of 15%. The addition of 1 m*M* HMBA to 100 n*M* 5azaC- and 1 n*M* RA-treated cultures significantly increased the colony-formation inhibition from only 2.6% and 7.0% to 46.4%, and 43.1%, respectively. Also, HMBA showed marked synergism with RA and 5azaC in inhibiting colony growth. The interaction indices (*I*) of HMBA + RA and HMBA + 5azaC were 0.013 and 0.009, respectively, at the same specific level of 15%. Moreover, the triple combination of RA + HMBA + 5azaC showed synergism in inhibiting both the colony formation ($I=0.7$) and colony growth ($I=0.4$) at the same specific level of 15%. Since RA, HMBA and 5azaC were effective when administered alone in phase I clinical trials of myeloid leukaemic patients, their synergistic combinations could provide shorter and less toxic courses of treatment in elderly myeloid leukaemic patients. *I* is <1, $=1$ or >1 in synergistic, additive or antagonistic interactions, respectively.

Key words: Azacytidine – Hexamethylene bisacetamide – HL-60 cells – Myelocytic leukaemia – Vitamin A

Introduction

Although the major clinical trials have achieved more than 75% remission rate in younger acute myeloid leukaemia (AML) patients with conventional cytotoxic chemotherapy, elderly patients receive little or no treatment because such intensive therapy is unsuitable (Rees 1989). As the majority of AML patients are over 65 years of age, there is a pressing need for another form of treatment which can effectively control the disease in this age group (Copplestone and Prentice 1988). Therapy with differentiation-inducing agents and/or cytostatic drugs provides a suitable alternative that does not incur an unreasonable risk of shortening the elderly person's life or make it unbearable (Sachs 1978; Hassan and Rees 1989a).

The identification of synergistic combinations of differentiation-inducing agents and/or cytostatic drugs that can induce maximum cytostatic effects on human myeloid leukaemic cells in vitro could provide effective therapeutic regimens for these elderly AML patients (Hassan 1988a). All-*trans*-retinoic acid (RA) inhibits the proliferation and induces the differentiation of several human myeloid leukaemic cell lines (Hassan 1988b), AML cells in primary culture (Breitman et al. 1981; Hassan and Rees 1988) and congenital agranulocytosis marrow myeloid progenitor cells in primary culture (Hassan et al. 1990), an effect that was enhanced by its combination with other agents in double and triple combinations (Hassan and Rees 1989b).

5-Azacytidine (5azaC) inhibits the proliferation and induces the differentiation of HL-60 human myeloid leu-

Abbreviations: AML, acute myeloid leukaemia; 5-azaC, 5-azacytidine; HMBA, hexamethylene bisacetamide; RA, all-*trans*-retinoic acid

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kaemic cells (Christman et al. 1983), an effect that was enhanced by its combination with cytarabine (Silverman et al. 1988).

Hexamethylene bisacetamide (HMBA) inhibits the proliferation and induces the differentiation of HL-60 cells (Collins et al. 1980), an effect that was not affected by inhibitory factors in AML serum (Hassan and Rees 1990a).

In order to determine new effective therapeutic regimens for elderly AML patients, the effect of combinations of RA, 5azaC and/or HMBA on the colony formation and growth of HL-60 cells was studied in an agar-capillary microclonogenic assay. The present study identified new potentially clinically useful synergistic combinations: HMBA + 5azaC, RA + 5azaC, RA + HMBA and RA + HMBA + 5azaC.

Materials and methods

HL-60 clonogenic microculture system. The clonogenic micro assays for HL-60 human myeloid leukaemic cells were carried out in agar-containing capillaries as described before (Maurer and Echarti 1990). HL-60 cells were cultured at a concentration of 2.5×10^4 cells/ml in glass capillaries containing RPMI-1640 medium supplemented with L-glutamine, 20% fetal calf serum and 0.18% agar in a final volume of 30 µl. Each of the differentiation-inducing agents at graded concentrations: all-*trans*-retinoic acid (1 n*M*–1 µ*M*), hexamethylene bisacetamide (0.5–4 m*M*) and 5-azacytidine (1 n*M*–1 m*M*) was added either alone or in combination with the others. Also, control cultures containing neither RA nor HMBA nor 5azaC were set up in conditions otherwise identical. For each assay point two capillaries were filled with 30 µl assay mixture and incubated for 7 days at 37° C in a humidified incubator under 7.5% CO_2. Colonies (aggregates of at least 20 cells) and the average number of cells per colony were counted in each capillary using a dissecting microscope on day 7.

Assessment of colony-formation inhibition and colony-growth inhibition. Dose/response curves of the percentage of surviving colonies relative to the number of control colonies on day 7 were plotted versus the concentrations of each drug when used alone or in combinations with each other. The colony growth was calculated by multiplying the number of colonies by the average number of cells per colony in each agar capillary. Dose/response curves of the percentage of colony growth relative to the colony growth in control cultures on day 7 were plotted versus the concentrations of each drug when used alone or in combinations with each other.

ED_{90} concentrations, i.e. the concentrations of RA, HMBA or 5azaC inducing a 90% inhibition of colony formation on day 7, were calculated from the logarithmic regression analysis of each dose/response curve using the cricket graph version 1.3 program on an Apple Macintosh computer. The results were represented as means ± standard errors of the mean of quadruplicate determinations and the statistical significance was determined using χ^2 and *t*-distribution tests.

Assessment of drug interactions between the differentiating agents. Drug-interaction studies have suffered from the application of some misplaced or inappropriate mathematical and statistical analyses, which have in a surprisingly large number of reports invalidated the conclusions drawn. The hyperplane theorem of Berenbaum (1989), which is derived from the isoboles constructed from dose/response curves, provides a general algebraic equation for calculating an interaction index (*I*) for combinations of two or more drugs:

$$I = (Ac/Ae) + (Bc/Be) + \ldots + (Nc/Ne).$$

This algebraic interaction index was calculated for the double or triple combinations of differentiating agents in the present study as described before. Where Ac, Bc, ..., Nc are doses of A, B, ... etc. in drug combinations producing a specified effect level and Ae, Be, ..., Ne are the doses of A, B, ... etc. that singly produce that effect. The effect level chosen in the present study was 15% colony-formation inhibition or colony-growth inhibition because this low level is better for the demonstration of synergy and because at high drug concentrations compound effects due to the secondary action of agents are more likely. The interaction index gives a measure of the nature and degree of interaction between these compounds, and equals 1 for additive interaction. <1 for synergistic interaction and >1 for antagonistic interaction. Statistical significance was estimated by calculation of the expected values for an additive interaction and determination of the observed − expected difference (χ^2) as described before (Berenbaum 1989).

Results

After 7 days of incubation, the numbers of HL-60 colonies and clonogenic cells in 1 n*M*–1 µ*M*) all-*trans*-retinoic-acid(RA)-treated cultures were 72.1 ± 15.5% and 78.2 ± 12.2%, respectively, of those in control cultures. Similar suppression of clonogenicity by RA was reported in day-7 agar cultures of HL-60 cells in petri dishes (Lübbert and Koeffler 1988).

Also, the numbers of HL-60 colonies and clonogenic cells in 1 µ*M*–1 m*M* 5-azacytidine(5-azaC)-treated cultures were 32.5 ± 13.1% and 61.5 ± 22.6%, respectively of those in control cultures. Similar suppression of clonogenicity by 5-azaC was reported in day-7 agar cultures of HL-60 cells (Christman et al. 1983).

Moreover, the numbers of HL-60 colonies and clonogenic cells in 0.5–4 m*M* HMBA-treated cultures were 61.7 ± 18.2% and 61.5 ± 18.2%, respectively, of those in control cultures on day 7.

The ED_{90} concentrations of RA, 5-azaC and HMBA were 127.6 n*M*, 39.4 µ*M* and 2.7 m*M*, respectively.

The addition of 1 m*M* HMBA to 100 n*M* 5azaC- or 1 n*M* RA-treated cultures significantly increased the colony-formation inhibition in HL-60 cells from only 2.6 ± 1.0% or 7.0 ± 3.9% to 46.4 ± 6.1% or 43.1 ± 13.9%,

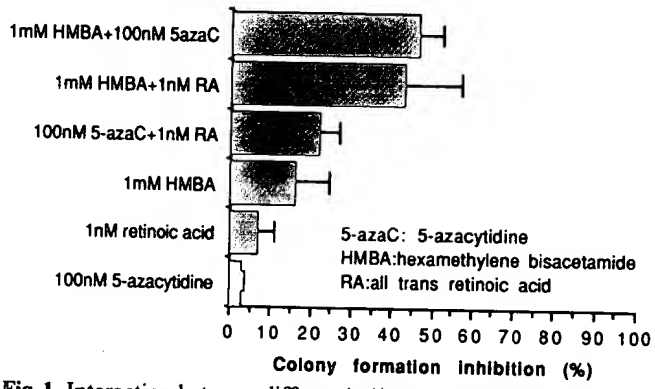

Fig. 1. Interaction between differentiation-inducing agents in inhibiting day-7 colony formation in HL-60 human myeloid leukaemic cells in clonogenic micro cultures. The results represent the means of quadruplicate determinations ± the standard error of the mean for each agar capillary. Colonies are aggregates of at least 20 cells after 7 days of incubation (see Materials and methods). HMBA, hexamethylene bisacetamide; RA, all-*trans*-retinoic acid; 5azaC, 5-azacytidine

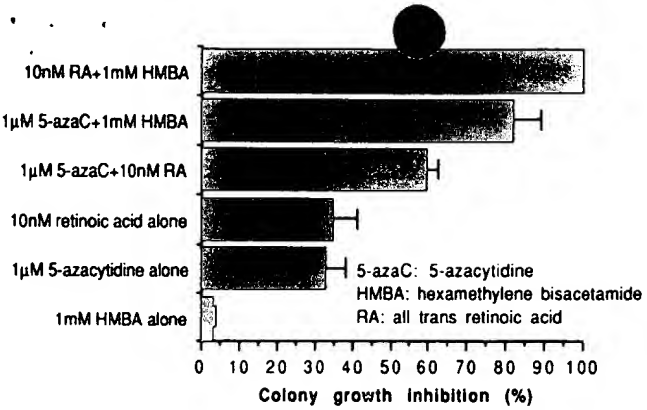

Fig. 2. Interaction between differentiation-inducing agents in inhibiting day-7 colony growth in HL-60 human myeloid leukaemic cells in clonogenic micro cultures. The results represent the means of quadruplicate determinations ± the standard error of the mean for each agar capillary. Colony growth was calculated by multiplying the number of colonies by the average number of cells per colony in each agar capillary (see Materials and methods).

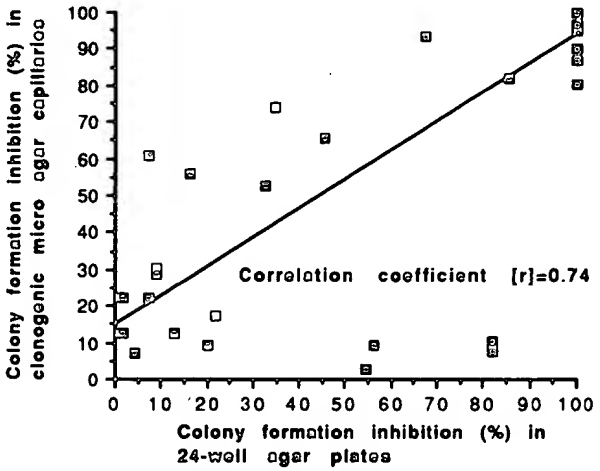

Fig. 3. Correlation of colony-formation inhibition in HL-60 cells between agar capillary and 24-well-plate agar cultures.

respectively, $P < 0.001$ (Fig. 1). Also, the addition of 1 m*M* HMBA to 1 μ*M* 5azaC- or 10 n*M* RA-treated cultures significantly increased the colony-growth inhibition in HL-60 cells from only 33.1 ± 5.2% or 34.7 ± 6.9% to 81.6 ± 7.6% or 100%, respectively, $P < 0.001$ (Fig. 2).

The present results were reproduced using 24-well-plate agar cultures for 7 days (correlation coefficient, $r = 0.74$) (Fig. 3).

The combinations HMBA + 5azaC, RA + HMBA and RA + 5azaC were significantly synergistic in inhibiting HL-60 colony formation ($P < 0.05$) and colony growth ($P < 0.001$) (Table 1). Similar synergism between HMBA and RA was recently reported in inducing differentiation of HL-60 cells in liquid suspension culture (Breitman and He 1990). Also, the triple combination of RA + HMBA + 5azaC showed significant synergism in inhibiting HL-60 colony formation ($P < 0.05$) and colony growth ($P < 0.001$) (Table 1).

Discussion

The combinations of differentiation-inducing agents and/or cytostatic drugs may cater for more than one type of abnormal activity in a complementary fashion at several levels: genomic, signal transduction, cell cycle, DNA topoisomerase etc. and thus synergistically can inhibit the proliferation of HL-60 cells (Hassan and Rees 1990b).

Synergistic combinations are important not only for limitation of toxicity but also because multiple drug combinations may better overcome the inherent heterogeneity in myeloid leukaemic patients. Also, synergy can be clinically important as it may increase the duration of effective drug levels in vivo (Berenbaum 1989). Thus, the present combinations could provide shorter and less toxic courses of treatment in elderly myeloid leukaemic patients.

The clinical response of patients with acute promyelocytic leukaemia (APL) to RA was outstanding (Meng-er et al. 1988; Castaigne et al. 1989; Wu et al. 1989). RA achieved a striking advantage over aggressive cytotoxic chemotherapy in APL patients. Instead of destroying leukaemic cells and causing the release of procoagulant factors from the azurophilic granules into the circulation, which usually results in an increased procoagulant activity and disseminated intravascular coagulopathy (DIC) in these APL patients, RA inhibited proliferation and induced differentiation, which led to a diminished procoa-

Table 1. Synergistic interactions between differentiation-inducing agents in inhibiting the colony formation and colony growth of HL-60 human myeloic leukaemic cells in clonogenic micro assays

Agent 1	Agent 2	Agent 3	Colony-formation inhibition[a]			Colony-growth inhibition[a]		
			Interaction index mean[b]	Observed – expected[c]	Statistical Significance, *P*	Interaction index mean[b]	Observed – expected[c]	Statistical significance, *P*
RA	HMBA	–	0.62	>0	<0.05	0.013	>0	<0.001
5-azaC	HMBA	–	0.83	>0	<0.05	0.009	>0	<0.001
RA	5-azaC	–	0.97	>0	<0.05	0.18	>0	<0.001
RA	5-azaC	HMBA	0.70	>0	<0.05	0.40	>0	<0.001

[a] Calculated by multiplying the number of colonies by the average number of cells per colony in each agar capillary

[b] Mean of quadruplicate determinations of interaction indices of combinations of four drug concentrations at the specific effect level of 15%. The interaction indices (*I*) were calculated by Berenbaum's general algebraic equation (Berenbaum 1989). *I* is <1, =1 or >1 in synergistic, additive or antagonistic interactions, respectively

[c] Calculated as described before (Berenbaum 1989). HMBA, hexamethylene bisacetamide (0.5–4 mM); RA. all-*trans*-retinoic acid (1 n*M*–1 μ*M*); 5-azaC, 5-azacytidine (1 n*M*–1 μ*M*)

gulant activity (Wijermans et al. 1989) and avoided or stopped DIC (Meng-er et. al. 1988; Castaigne et al. 1989; Wu et al. 1989).

Also, RA significantly increased the remission duration both in AML children when given in a high dose of 50000 IU/day after complete remission (Lie et al. 1988) and in AML adults when given in a dose of 20 mg/day as a maintainence therapy (Curtis et al. 1989). In these two clinical studies, therapy was well-tolerated without any clinical or biochemical side-effects for up to 4 and 2 years, respectively.

The present results are of potential significance to the application of RA in combination with other differentiating agents in clinical trials for myeloid leukaemic patients. The synergistic effect of successive courses of short doses of RA with 5azaC and/or HMBA may be an efficient way to minimise any potential toxicity incurred by the prolonged exposure to them. Also, the successive courses of short doses of RA with 5azaC and/or HMBA could sustain a long clinical remission and thus overcome the problem of sustaining the response seen in clinical studies of RA as a single-agent therapy (Meng-er et al. 1988; Castaigne et al. 1989; Wu et al. 1989).

The synergistic combinations of nanomolar concentrations of RA with 5azaC and/or HMBA achieved a very high proliferation-inhibition rate (Fig. 2) and therefore warrants a clinical trial for elderly myeloid leukaemic patients.

Also, the combination of cyclophosphamide with *N*-methylformamide, which is a polar compound like HMBA, increased their in vivo activity without any concomittant increase in their reversible hepatoxicity (Langdon et al. 1985). Therefore, the pharmacologically achievable in vivo concentrations of the synergistic combinations of HMBA with RA and/or 5azaC can be administered clinically without any significant additive hepatotoxicity in myeloid leukaemic patients. Moreover, five phase I clinical trials of HMBA showed no myelosuppression and three more clinical trials of HMBA in myelodysplastic patients are now in progress (Egorin 1988). Human pharmacokinetic studies have revealed that by using HMBA at doses $\leqq 24\ g\ m^{-2}\ day^{-1}$ in a 5-day continuous infusion schedule, a steady plasma concentration of 1 m*M* can be maintained for this period (Egorin 1988). Therefore, treatment with $24\ g\ m^{-2}\ day^{-1}$ continuous infusion HMBA with RA and/or 5azaC could be an effective remission-induction therapy for elderly AML patients.

Drug resistance is a major obstacle in the treatment of refractory myeloid leukaemic patients and may be due to the presence of dominant resistant populations of blast cells (Asano et al. 1989). The treatment of myeloid leukaemic cells with a combination of a differentiating agent and a low concentration of an antileukaemic drug, e.g. cytarabine or actinomycin-D, was effective in suppressing the emergence of differentiation-resistant cells (Kasukabe et al. 1987). The present synergistic combinations of micromolar concentrations of the antileukaemic drug (5azaC) with RA and/or HMBA achieved a very high proliferation-inhibition rate (Fig. 2). 5azaC has been shown to be effective when administered alone in the clinical treatment of acute leukaemia (Karon et al. 1973; Saiki et al. 1978). Therefore, the present synergistic combinations of 5azaC with RA and/or HMBA could provide the basis for an effective maintenance therapy for myeloid leukaemic patients in remission. This maintenance therapy could presumably increase the remission duration in these patients by preventing the regrowth of the small number of residual leukaemic cells remaining after remission-induction therapy. Clinical studies on the basis of these in vitro findings may support or disprove our assumptions.

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Inhibitors of arachidonic acid metabolism potentiate tumour necrosis factor-α-induced apoptosis in HL-60 cells

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Abstract

We investigated whether and how could various modulators of arachidonic acid metabolism affect apoptosis induced by tumour necrosis factor-α (TNF-α) in human myeloid leukaemia HL-60 cells. These included arachinonyltrifluoromethyl ketone ($AACOCF_3$; cytosolic phospholipase A_2 inhibitor), indomethacin (cyclooxygenase inhibitor), MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid; 5-lipoxygenase-activating protein inhibitor), nordihydroguaiaretic acid (general lipoxygenase inhibitor), and arachidonic acid itself. Incubation of HL-60 cells with nordihydroguaiaretic acid resulted in apoptosis and it was characterised by mitochondria membrane depolarisation, release of cytochrome *c* from mitochondria into cytosol and activation of caspase-3. Indomethacin and nordihydroguaiaretic acid synergistically potentiated TNF-α-induced apoptosis, while arachidonic acid, $AACOCF_3$ and MK-886 did not modulate its effects. Furthermore, indomethacin potentiated apoptosis in cells treated with a differentiating agent, *all-trans* retinoic acid, which induces resistance to TNF-α. However, the observed effects were probably not associated either with the cyclooxygenase- or lipoxygenase-dependent activities of indomethacin and nordihydroguaiaretic acid, respectively. Since indomethacin may reportedly activate peroxisome proliferator-activated receptors (PPARs), the effects of specific ligands of PPARs on apoptosis were studied as well. It was found that selective PPARs ligands had no effects on TNF-α-induced apoptosis. The findings suggest that arachidonic acid metabolism does not play a key role in regulation of apoptosis induced by TNF-α in the present model. Nevertheless, our data raise the possibility that indomethacin could potentially be used to improve the treatment of human myeloid leukaemia.

Keywords: Retinoic acid, *all-trans*; TNF-α (tumor necrosis factor-α); Fas; Arachidonic acid; Lipoxygenase; Cyclooxygenase; PPAR (Peroxisome proliferator-activated receptor)

1. Introduction

Inappropriate cell survival has been linked to the development of various diseases, including human malignancies. It is controlled by programmed cell death (apoptosis), an active process that is critical for the homeostasis of tissues (Steller, 1995). Apoptosis is characterised by membrane blebbing, cytoplasmic, nuclear and chromatin condensation, activation of caspases, and DNA cleavage into multiples of intranucleosomal fragments (Kerr et al., 1972; Schulze-Osthoff et al., 1998). It can be induced by a number of stimuli, including the deprivation of survival factors, cell damaging stress, chemotherapy, and signals through death receptors (Jarpe et al., 1998). Death receptors, belonging to the tumour necrosis factor (TNF) receptor family, induce apoptosis through pathways that share many similarities, including recruitment of death domain-containing adapter proteins and activation of the caspase cascade via caspase-8 (Ashkenazi and Dixit, 1998; Jarpe et al., 1998; Schulze-Osthoff et al., 1998).

The action of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) is mediated by its receptors, TNF receptor 1 (CD120a, p55 receptor) and TNF receptor 2 (CD120b, p75 receptor), the former belonging among death receptors. During recent years, four main groups of

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signalling molecules have been identified downstream of TNF receptor 1-caspases, phospholipases, mitogen activated protein kinases and the NF-κB signalling cascade (Wallach et al., 1999). TNF-α has been shown to induce release of arachidonic acid through activation of cytosolic phospholipase A_2; however, the role that this enzyme plays in mediating TNF-α-induced cell death has not been completely elucidated (Leslie, 1997; Wallach et al., 1999). Arachidonic acid release leads to the formation of a series of metabolites, including prostaglandins generated through cyclooxygenases, hydroxyeicosatetraenoic acids, lipoxins and leukotrienes generated through lipoxygenases and the products of the *P*450-monooxygenase system (Shimizu and Wolfe, 1990).

Thus, the arachidonic acid cascade generates a family of bioactive lipids that modulate diverse physiological and pathological responses, including tumour growth and promotion (Ara and Teicher, 1996). We and others have shown that arachidonic acid products, namely those of the 5-lipoxygenase pathway, play an important role in myelopoiesis and the regulation of proliferation and differentiation of human leukaemic cells (Hofmanová et al., 1998; Kozubík et al., 1997; Stenke et al., 1994). However, their role in the regulation of apoptosis in leukaemic cells is less clear.

It has been suggested that tumour growth, promotion and metastasis can be modulated by inhibition of production of arachidonic acid metabolites generated through lipoxygenase and cyclooxygenase pathways (Ara and Teicher, 1996). Recently, a number of both lipoxygenase and cyclooxygenase inhibitors, including non-steroidal anti-inflammatory drugs (NSAIDs), have been reported to induce apoptosis in various types of cancer cells, including cells of myeloid origin (Anderson et al., 1998; Bellosillo et al., 1998; Datta et al., 1999; Dittmann et al., 1998; Ghosh and Myers, 1998; Chan et al., 1998; Tang et al., 1996; Zhang et al., 2000). However, the role of various types of inhibitors of arachidonic acid metabolism is unclear, since it has been reported that they may even protect from apoptosis induced by TNF-α (Hepburn et al., 1987; Chang et al., 1992; O'Donnell et al., 1995).

In the present study, we investigated whether and how could various modulators of arachidonic acid metabolism affect apoptosis induced by TNF-α or an agonist antibody against Fas (another type of death receptor) in human myeloid leukaemia HL-60 cells. These included arachidonyltrifluoromethyl ketone ($AACOCF_3$; cytosolic phospholipase A_2 inhibitor), indomethacin (cyclooxygenase inhibitor), MK886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid; 5-lipoxygenase-activating protein inhibitor), nordihydroguaiaretic acid (general lipoxygenase inhibitor when used at concentrations lower than those inhibiting cyclooxygenase), and arachidonic acid itself. It has been demonstrated that *all-trans* retinoic acid, an inducer of granulocytic differentiation in myeloid leukaemia cells, can efficiently inhibit TNF-α-induced apoptosis (Kikuchi et al., 1996; Vondráček et al., 2001). Therefore, we investigated the effects of indomethacin, which was found to significantly potentiate the TNF-α-induced programmed cell death, on suppression of apoptosis observed during *all-trans* retinoic acid-induced differentiation. Finally, since indomethacin was found to synergistically enhance TNF-α-induced apoptosis in relatively high concentrations in the present study and it has been reported to activate peroxisome proliferator-activated receptors (PPARs) (Lehmann et al., 1997), the effects of specific ligands of PPAR-α and -γ on apoptosis were studied as well.

2. Materials and methods

2.1. Cells

Human myeloid leukaemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) and maintained in a humidified incubator at 37 °C in 5% CO_2. Exponentially growing cells were plated into Petri dishes at 2×10^5 cells per ml 1 h prior to application of inhibitors. For *all-trans* retinoic acid treatment, exponentially growing cells were seeded directly into medium supplemented with 1 μM *all-trans* retinoic acid.

2.2. Reagents

Human recombinant TNF-α, indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), nordihydroguaiaretic acid, arachidonic acid, RPMI-1640 medium, RNAse A, propidium iodide, secondary anti-murine IgG antibody conjugated with horse radish peroxidase and *all-trans* retinoic acid were purchased from Sigma (St. Louis, MO, USA). Wy-14,643 (4-chloro-6-[2,3-xylidino]-2-pyrimidinylthioacetic acid; PPAR-α ligand) and ciglitazone ((±)-5-[4-(1-methylcyclohexylmethoxy)-benzyl]thiazolidine-2,4-dione]; PPAR-γ ligand) were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). MK-886 was a kind gift from Merck (Canada). $AACOCF_3$ was from Calbiochem (San Diego, CA, USA). Anti-human Fas CH-11 antibody was from Immunotech (Marseille, France). Murine monoclonal anti-5-lipoxygenase and anti-caspase-3 antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Murine monoclonal anti-cytochrome *c* antibody was from PharMingen (San Diego, CA, USA). Rabbit polyclonal anti-cyclooxygenase-2 antibody and murine monoclonal anti-cytosolic phospholipase A_2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-rabbit IgG antibody conjugated with horse radish peroxidase was purchased from

Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum was from PAN Systems (Nürnberg, Germany). 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was from Fluka (Buchs, Switzerland). MOWIOL® 40-88 was obtained from Aldrich (Milwaukee, WI, USA).

2.3. Induction and detection of apoptosis

Cells were incubated with selected inhibitors of arachidonic acid metabolism or PPAR ligands for 1 h prior to 16-h incubation with human recombinant TNF-α (0.001–10 ng/ml final concentration) or CH-11 mouse monoclonal antibody (100 ng/ml final concentration), which was reported to induce apoptosis by crosslinking Fas (Yonehara et al., 1989). Inhibitors of arachidonic acid metabolism were prepared as stock solutions in ethanol and stored at −20 °C. PPAR ligands and $AACOCF_3$ were dissolved in dimethyl sulfoxide and stored at −80 °C. Arachidonic acid was dissolved in ethanol and stored under nitrogen atmosphere at −80 °C. Following the incubation with apoptosis inducers, cells were harvested and prepared for DNA labelling with propidium iodide or DAPI, as follows.

For propidium iodide staining, cells were washed once with phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. Fixed cells were washed twice with PBS and low molecular weight DNA was extracted with citric acid buffer (Gong et al., 1994). Cells were then resuspended in PBS containing 20 μg/ml propidium iodide and 5 Kunitz U/ml RNAse A and incubated for 30 min at room temperature. Cells were analysed using a FACS©Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 15,000 events was collected per sample.

To verify the results of flow cytometry, nuclear morphology was examined by fluorescence microscopy. Cells (5×10^5) were resuspended with 50 μl of methanol containing 2 μg/ml DAPI (final concentration) and incubated for 30 min at room temperature. After incubation, cells were centrifuged, mixed with 20 μl of MOWIOL solution and mounted for counting with a fluorescence microscope. A minimum of 200 cells was counted per sample.

2.4. Western blot analysis

HL-60 cells were treated with selected compounds or their combination with TNF-α and CH-11 antibody for the time indicated. Cells were washed with PBS, cell pellets were lysed using sodium dodecyl sulfate (SDS) lysis buffer, and 30 μg of total protein per sample was separated on 12% (for detection of caspase-3 cleavage) or on 7.5% (for detection of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2 expression) polyacrylamide gel and transferred onto a polyvinyldifluoride membrane. After incubation with primary and secondary antibodies, detection was performed using the ECLPlus Western blotting detection system (Ammersham Pharmacia Biotech, Little Chalfont, UK).

For detection of cytochrome *c* release, cells were fractionated using the modified method described by Chen et al. (2000). Cells were twice washed with PBS and sonicated (3 × 5 s on ice) in buffer containing 20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose and protease inhibitors. The

Table 1
Effects of inhibitors of arachidonic acid metabolism on TNF-α-induced apoptosis in HL-60 cells

Treatment	%Apoptotic cells			
	None	TNF-α (0.1 ng/ml)	TNF-α (1 ng/ml)	TNF-α (10 ng/ml)
None	10.0 ± 2.1	13.4 ± 3.6	23.1 ± 4.6	31.0 ± 7.5
AA (10 μM)	8.0 ± 1.2	7.9 ± 3.4	18.0 ± 7.1	24.5 ± 7.8
AA (20 μM)	7.5 ± 4.2	9.8 ± 4.6	20.8 ± 6.9	24.8 ± 4.9
AA (40 μM)	13.8 ± 7.5	19.5 ± 8.5	26.2 ± 10.2	31.5 ± 11.7
$AACOCF_3$ (10 μM)	8.3 ± 3.6	9.5 ± 5.7	20.3 ± 4.0	26.8 ± 3.8
$AACOCF_3$ (20 μM)	7.8 ± 3.0	10.3 ± 5.0	21.5 ± 6.4	28.5 ± 4.4
$AACOCF_3$ (40 μM)	7.3 ± 3.9	11.8 ± 4.4	21.8 ± 4.4	30.0 ± 3.9
MK-886 (1 μM)	13.3 ± 2.2	18.0 ± 3.6	27.8 ± 4.6	33.3 ± 7.3
MK-886 (5 μM)	14.0 ± 3.2	20.3 ± 3.4	28.0 ± 3.2	35.3 ± 5.0
MK-886 (10 μM)	14.5 ± 2.5	24.8 ± 6.4	31.8 ± 4.4	40.3 ± 3.8
NDGA (1 μM)	11.0 ± 4.1	13.4 ± 4.5	22.5 ± 3.7	31.3 ± 5.4
NDGA (5 μM)	21.8 ± 7.1[a]	21.3 ± 7.1[a]	34.0 ± 8.6[a]	38.8 ± 7.8[a]
NDGA (10 μM)	31.0 ± 6.9[a]	28.8 ± 3.4[a]	35.0 ± 5.7[a]	43.5 ± 6.8[a]
Indomethacin (10 μM)	12.0 ± 2.2	19.0 ± 5.3	32.3 ± 6.5	46.3 ± 8.8
Indomethacin (50 μM)	14.0 ± 3.7	28.8 ± 4.2[a]	40.3 ± 6.7[a]	51.8 ± 4.4[a]
Indomethacin (100 μM)	17.0 ± 2.9[a]	45.8 ± 3.3[a]	50.5 ± 7.6[a]	60.0 ± 7.3[a]

Cells were treated with TNF-α in the presence or absence of inhibitors in RPMI 1640 medium under standard cultivation conditions for 16 h. The percentage of apoptotic cells was determined by flow cytometry. All values represent means ± S.D. of at least three independent experiments. AA—arachidonic acid.

[a]A significant difference from the corresponding control group (without addition of inhibitors, $P < 0.05$). Differences were analysed using Mann–Whitney *U*-test and Kruskal–Wallis ANOVA.

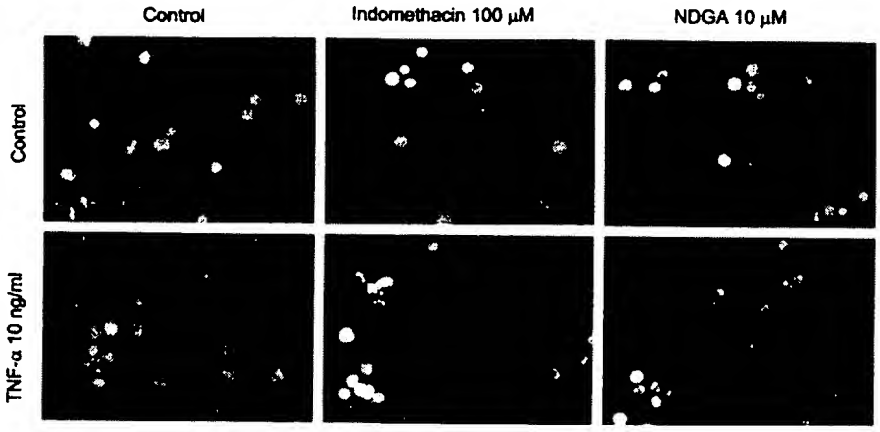

Fig. 1. Indomethacin and nordihydroguaiaretic acid (NDGA) potentiate TNF-α-induced apoptosis in HL-60 cells. Cells were stained with DAPI and observed by fluorescence microscopy. Results are representative of three independent experiments.

homogenates were centrifuged at 750 × *g* for 5 min, and the supernatant was then centrifuged at 10,000 × *g* for another 5 min. The mitochondria-containing pellet was designated P10 and the supernatant was subjected to further ultracentrifugation at 100,000 × *g* for 60 min. The resulting supernatant represented the cytosolic fraction. Proteins in the supernatants were concentrated with 5–10% trichloroacetic acid. Both the supernatants and P10 were dissolved in Laemmli sample buffer (50 mM TRIS pH 6.8, 2% SDS, 10% glycerol). Following the quantification of proteins with Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), all lysates were diluted to same protein concentration (1 mg/ml), and 1% β-mercaptoethanol and 0.1% bromophenol blue were added into lysates. Proteins were separated on polyacrylamide gel, transferred onto a polyvinyldifluoride membrane and probed with antibody against cytochrome *c* followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Detection was performed using the ECLPlus Western blotting detection system.

2.5. Detection of mitochondrial membrane potential

The variation of mitochondrial transmembrane potential during nordihydroguaiaretic acid and indomethacin treatment was studied using tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Eugene, OR, USA) (Loew et al., 1994). Cells were washed twice with Hanks' balanced salt solution (HBSS), approximately 10^6 cells was resuspended in 100 nM of TMRE in HBSS and

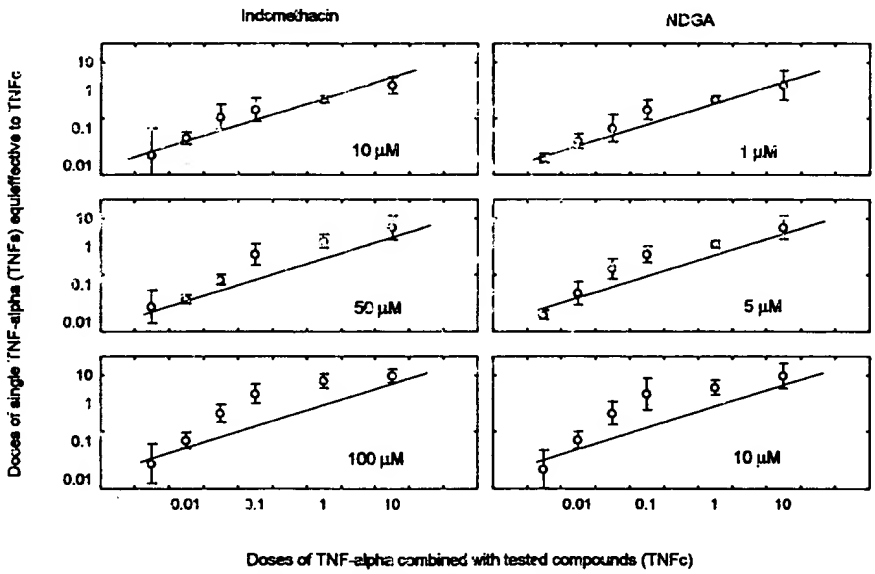

Fig. 2. Effects of combinations of TNF-α plus indomethacin or nordihydroguaiaretic acid (NDGA) on apoptosis of HL-60 cells. Cells were stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Comparison of single TNF-α doses (TNFs) that are equieffective with the concentration of TNF-α combined with selected compound (TNFc). Ranges (I) represent 95% confidence intervals of respective mean value (○). No overlap of confidence interval with the diagonal additivity line indicates a significant potentiation of TNF-α induced apoptosis by a respective agent (synergy effect). Doses of TNF-α (ng/ml) were expressed in log units.

incubated for 20 min at room temperature in the dark. At the end of the incubation period, cells were washed with HBSS, resuspended in a total volume of 500 μl and analysed on FACS©Calibur flow cytometer (585/42 band pass filter). Data were evaluated as median of fluorescence of living cells gated by forward scatter versus side scatter.

2.6. Statistics

Data were expressed as means ± S.D. for at least three independent repeats and analysed by the nonparametric Mann–Whitney *U*-test and Kruskal–Wallis analysis of variance (ANOVA). To study interactive effects between TNF-α and effective compounds (nordihydroguaiaretic acid, indomethacin), an isobolographic approach based on the analysis of the equieffective quantities of agents in the mixture was used as described (Gessner, 1988). A P value of less than 0.05 was considered significant. All analyses were carried out using GraphPad Prism™ and MS-Excel™ software packages.

3. Results

3.1. Indomethacin and nordihydroguaiaretic acid significantly potentiate TNF-α-induced apoptosis

To examine effects of various inhibitors of arachidonic acid metabolism on sensitivity to TNF-α, changes in the subG$_1$ population in propidium iodide-stained cells, after extraction of low-molecular DNA with citric acid buffer, were studied. Table 1 gives a description of the observed effects. TNF-α induced apoptosis in HL-60 cells in a dose-dependent manner. Arachidonic acid, AACOCF$_3$ and MK-886 neither induced apoptosis in HL-60 cells nor potentiated the effects of TNF-α. On the other hand, nordihydroguaiaretic acid and indomethacin induced apoptosis when used at higher concentrations ($P < 0.05$). Both compounds were found to significantly potentiate induction of apoptosis by TNF-α ($P < 0.05$). The potentiation of TNF-α-mediated apoptosis by nordihydroguaiaretic acid and indomethacin was confirmed by morphological analysis of DAPI-stained cells, which revealed a significantly higher percentage of nuclei with typical apoptotic features in cells treated with either inhibitor plus TNF-α when compared to cells treated with TNF-α alone (Fig. 1).

Isobolographic analysis revealed that TNF-α combined with either indomethacin or nordihydroguaiaretic acid induced apoptosis in excess of a simple dose-additive manner. Nearly all of the tested concentrations of TNF-α combined with indomethacin (inhibitor concentrations, 50 and 100 μM) or NDGA (inhibitor concentrations, 5 and 10 μM) were significantly lower than the corresponding equieffective doses of single TNF-α (Fig. 2). Significant departures from zero interaction (dose-additive effect, the diagonal line in Fig. 2) were detected ($P < 0.05$), indicating a clear synergy in the action of TNF-α and the respective compounds.

3.2. Nordihydroguaiaretic acid induces mitochondrial depolarisation, cytochrome c release and caspase-3 cleavage in HL-60 cells

Since both nordihydroguaiaretic acid and indomethacin were found to induce cell death in HL-60 cells, we attempted to further characterise the mechanisms underlying

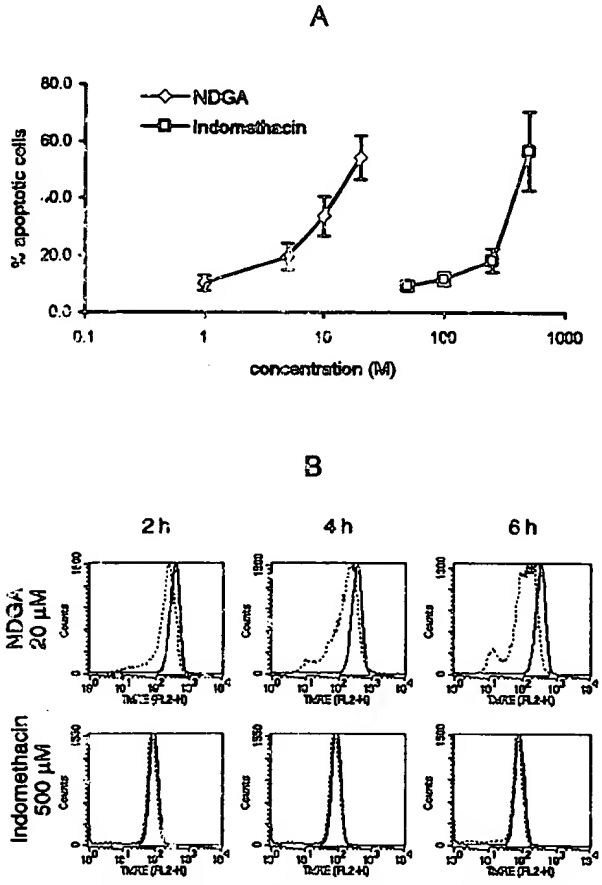

Fig. 3. Both indomethacin and nordihydroguaiaretic acid (NDGA) induce cell death in HL-60 cells in a dose-dependent manner, while only NDGA induces mitochondrial membrane depolarisation. (A) Cells were treated with indicated concentrations of indomethacin and NDGA, stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Points represent means ± S.D. from three independent experiments. (B) Cells were incubated with 20 μM NDGA or 500 μM indomethacin for the time indicated, the cells were collected and stained with TMRE (100 nM) for 20 min and analysed on FACS® Calibur flow cytometer. The histograms show mitochondrial potential of living cells, gated on forward versus side scatter to exclude debris and dead cells. The results are representative of at least three independent experiments. The decrease in TMRE fluorescence demonstrates the loss of membrane potential in NDGA-treated group (dot lines), while no decrease is observable the vehicle-treated control group (full lines).

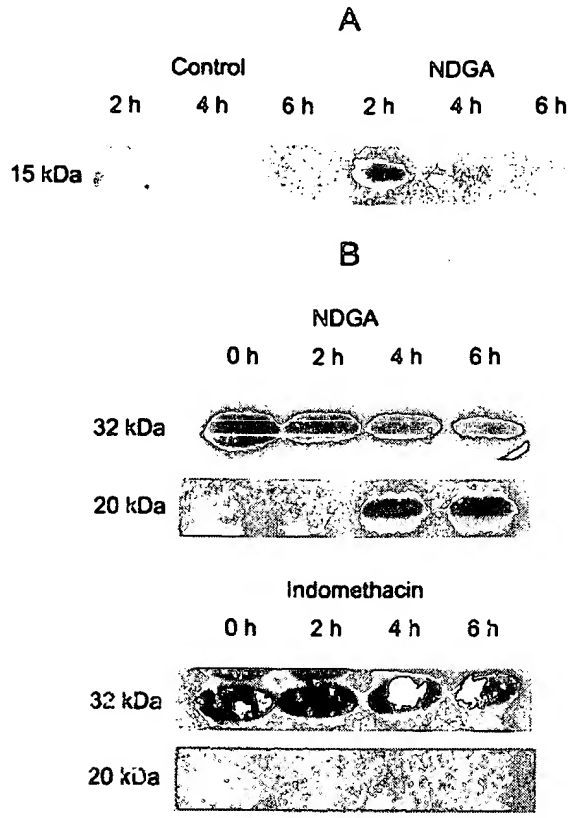

Fig. 4. Nordihydroguaiaretic acid (NDGA) induces cytochrome *c* release and caspase-3 activation. (A) Western blot analysis shows presence of cytochrome *c* in cytosolic fraction from NDGA-treated cells, indicating the release of cytochrome *c* from mitochondria. Cells were treated with 20 μM NDGA for the time indicated, fractionated, and Western blotting of cytosolic fraction was performed as described in Materials and methods. The Western blot is representative of two independent experiments. (B) Western blot analysis of caspase-3 cleavage upon NDGA and indomethacin treatment. Cells were treated with 20 μM NDGA or 500 μM indomethacin for the time indicated, lysed, and the lysate was used to perform Western blotting as described in Materials and methods; 30 μg total protein was loaded per lane. The results are representative of four independent experiments.

the apoptotic effects of these compounds. Mitochondrial membrane depolarisation and activation of caspase-3, cysteine protease play an important role in the process of apoptosis (Kroemer and Reed, 2000; Tyas et al., 2000). Compared to nordihydroguaiaretic acid, indomethacin was markedly cytotoxic only when used at very high concentrations (250 and 500 μM) (Fig. 3A). Nordihydroguaiaretic acid (20 μM) was found to induce loss of mitochondrial membrane potential (Fig. 3B) and cytochrome *c* release was detected already after 2 h of treatment (Fig. 4A). Incubation of HL-60 cells with nordihydroguaiaretic acid led to caspase-3 processing after 4 and 6 h of treatment, documented by the appearance of 20-kDa fragment (Fig. 4B). Contrary to that, we did not observe mitochondrial membrane depolarisation or caspase-3 cleavage in indomethacin-treated cells (Figs. 3B and 4B, respectively).

3.3. Selective inducers of PPARs do not alter TNF-α-induced apoptosis

Since NSAIDs have been shown to act as ligands of PPARs when present at relatively high concentrations, we investigated whether ligand-induced activation of these receptors could potentiate TNF-α-induced apoptosis. Two specific ligands for PPAR-α (Wy-14,643) and PPAR-γ (ciglitazone) were used in combination with TNF-α. However, neither of the compounds induced apoptosis or potentiated the effect of TNF-α in concentrations up to 50 μM (Table 2). The findings were confirmed by morphological analysis (data not shown, $n = 3$).

3.4. Combined treatment of HL-60 cells with both TNF-α and indomethacin can overcome all-trans retinoic acid-induced inhibition of apoptosis

In another set of experiments, the effects of combined treatment with indomethacin and TNF-α on apoptosis of cells treated with 1 μM *all-trans* retinoic acid were evaluated. The application of *all-trans* retinoic acid significantly inhibited TNF-α-induced apoptosis, as documented by a decrease in the subG$_1$ population (Fig. 5). However, indomethacin (100 μM) in combination with TNF-α was able to induce apoptosis to a similar extent as TNF-α

Table 2
Effects of PPAR ligands on TNF-α-induced apoptosis in HL-60 cells

Treatment	%Apoptotic cells			
	None	TNF-α (0.1 ng/ml)	TNF-α (1 ng/ml)	TNF-α (10 ng/ml)
None	9.25 ± 5.4	12.3 ± 8.1	31.0 ± 5.7	45.0 ± 7.4
Wy14,643 (10 μM)	9.0 ± 4.9	12.0 ± 5.5	27.8 ± 4.3	43.0 ± 7.9
Wy14,643 (50 μM)	12.0 ± 5.7	13.0 ± 5.7	30.5 ± 3.5	45.5 ± 12.0
Ciglitazone (10 μM)	9.3 ± 4.4	10.8 ± 5.7	22.8 ± 4.8	33.3 ± 6.6
Ciglitazone (50 μM)	14.5 ± 9.2	17.0 ± 9.9	24.0 ± 7.1	33.5 ± 5.1

Cells were treated with TNF-α in the presence or absence of PPAR ligands in RPMI 1640 medium under standard cultivation conditions for 16 h. The percentage of apoptotic cells was determined by flow cytometry. All values represent means ± S.D. of at least three independent experiments. [a]A significant difference from the corresponding control group (without addition of inhibitors, $P < 0.05$). Differences were analysed using Mann–Whitney *U*-test and Kruskal–Wallis ANOVA.

alone, although the combined effect was lower than in untreated cells (Fig. 5).

3.5. Indomethacin does not sensitise HL-60 cells to induction of apoptosis mediated by Fas

To further investigate the effect of indomethacin, we tested its effect on apoptosis mediated by Fas, a member of the TNF receptor superfamily. HL-60 cells were resistant to Fas-mediated apoptosis induced by Fas-crosslinking antibody at concentrations of 0.1–1 μg/ml (data not shown). The addition of indomethacin did not lead to sensitisation of cells towards CH-11 antibody as determined by flow cytometry (Fig. 6). These results were confirmed by fluorescence microscopy (data not shown, $n = 3$).

3.6. Nordihydroguaiaretic acid and indomethacin have no effect on expression of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2 in HL-60 cells

Since both cyclooxygenase- and lipoxygenase-independent effects have been suggested to be involved in nordihydroguaiaretic acid- and indomethacin-induced apoptosis (Biswal et al., 2000; Zhang et al., 2000), we studied effects of nordihydroguaiaretic acid and indomethacin on expression of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2. The inhibitors were applied to cells either alone or in combination with TNF-α (or CH-11 antibody in case of indomethacin). Undifferentiated HL-60 cells did not express 5-lipoxygenase protein and its expression was not induced by any of treatments (Fig. 7). This was confirmed by high performance liquid chromatography analysis, as we did not find detectable levels of 5-hydroxyeicosatetraenoic acid in an assay detecting 5-lipoxygenase activity (data not shown). None of the treatments significantly affected expression of cyclooxygenase-2 or cytosolic phospholipase A_2 in HL-60 cells (Fig. 7).

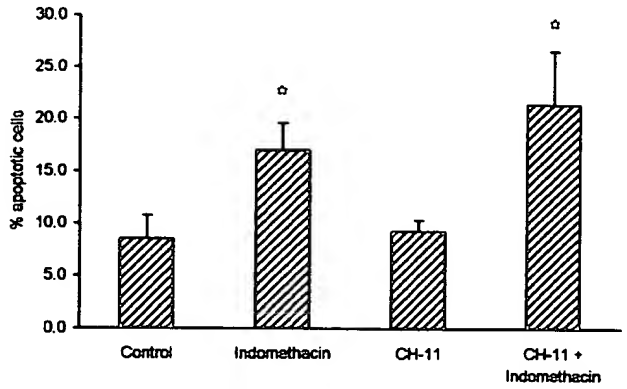

Fig. 6. Effects of combination of anti-human Fas murine monoclonal antibody CH-11 (100 ng/ml) with indomethacin (100 μM) on apoptosis of HL-60 cells. Cells were stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Bars represent means ± S.D. from a minimum of three independent experiments. * Significant difference from the control group ($P < 0.05$) as determined by Mann–Whitney *U*-test.

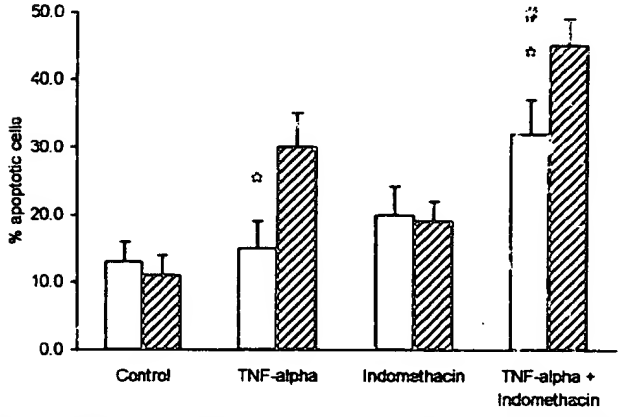

Fig. 5. Effects of combination of TNF-α (1 ng/ml) with indomethacin (100 μM) on apoptosis of untreated HL-60 cells (shaded bars) and cells treated with 1 μM *all-trans* retinoic acid (open bars). Cells were stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Bars represent means ± S.D. from a minimum of three independent experiments. * Significant difference from the respective control group ($P < 0.05$), # Significant difference from the group treated with both TNF-α and *all-trans* retinoic acid ($P < 0.05$), as determined by Mann–Whitney *U*-test.

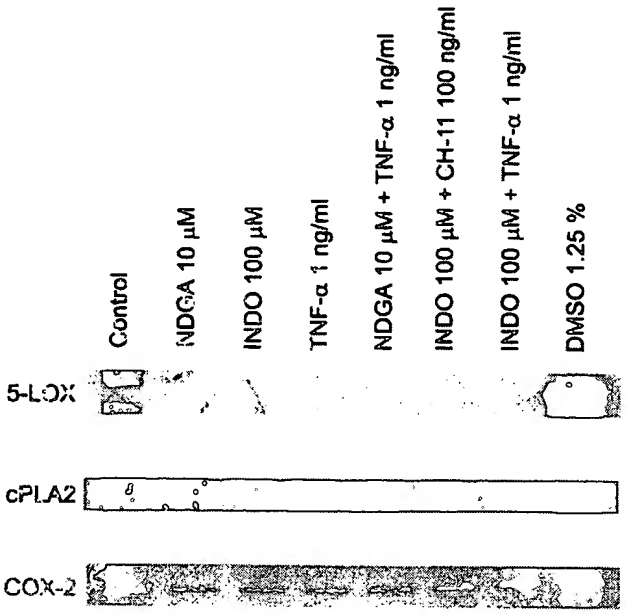

Fig. 7. Neither nordihydroguaiaretic acid (NDGA), nor indomethacin (INDO) induce 5-lipoxygenase (5-LOX) expression or affect the expression of cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 (cPLA2) in HL-60 cells. Western blot analysis of protein expression was carried out as described in Materials and methods and the results are representative of at least three independent experiments. Cells were treated with inhibitors and/or TNF-α (or CH-11) for 16 h; 30 μg of total protein was loaded per lane. Differentiated HL-60 cells (incubated for 96 h with 1.25% dimethyl sulfoxide—DMSO) were used as a positive control for 5-LOX expression.

4. Discussi n

Arachidonic acid has been reported to induce apoptosis in various cell types including myeloid leukaemia cell lines (Finstad et al., 1994, 1998; Rizzo et al., 1999; Surette et al., 1999) and it has been suggested to be an important mediator in regulation of sensitivity to TNF-α-induced apoptosis (De Valck et al., 1998; Hayakawa et al., 1993; Wissing et al., 1997; Wu et al., 1993). Various mechanisms, including lipid peroxidation and ceramide generation could play a role in arachidonic acid-induced programmed cell death (Jayadev et al., 1994). Contrary to that, it has been reported that arachidonic acid may even potentiate HL-60 proliferation in concentrations up to 80 μM (Liu and Levy, 1997), and that it may enhance tumour cell growth by promoting cell proliferation and by suppressing apoptosis (Tang et al., 1997). In the present study, arachidonic acid neither induced apoptosis nor modulated the effects of TNF-α. The discrepancies could be explained by the fact that cells are often incubated with high amounts of arachidonic acid (up 120 μM) for long time periods (up to 3 days in culture) in order to induce programmed cell death (Finstad et al., 1994, 1998), or by use of different cellular models.

Arachidonic acid has been suggested to participate in regulation of TNF-α-induced apoptosis, since overexpression of cytosolic phospholipase A_2, a principle enzyme responsible for TNF-α-induced arachidonic acid release, might increase sensitivity of cells to TNF-α cytotoxicity (Hayakawa et al., 1993), while inhibitors of cytosolic phospholipase A_2 block apoptosis induced by this cytokine (Wissing et al., 1997; Wu et al., 1998). However, we found that inhibition of arachidonic acid release by $AACOCF_3$ had no significant effects on TNF-α-induced cell death. Again, different cellular models could be partially responsible for the observed discrepancies, as some of the above-mentioned studies were performed in L929 murine fibrosarcoma cells. Moreover, inhibition of cytosolic phospholipase A_2 in some studies only partially blocked TNF-α-induced apoptosis. Neither indomethacin nor nordihydroguaiaretic acid, two compounds enhancing TNF-α-induced apoptosis, affected the expression of cytosolic phospholipase A_2. The findings suggest that arachidonic acid release does not play a key role in induction of programmed cell death by TNF-α in HL-60 cells.

The effects of various lipoxygenase and cyclooxygenase inhibitors on programmed cell death are unclear, since they have been reported both to induce and to reduce apoptosis in different cell types (Anderson et al., 1998; Bellosillo et al., 1998; Datta et al., 1999; Dittmann et al., 1998; Ghosh and Myers, 1998; Hepburn et al., 1987; Chang et al., 1992; O'Donnell et al., 1995). In the present study, nordihydroguaiaretic acid induced apoptosis in HL-60 cells and it synergistically potentiated the effects of TNF-α. Nordihydroguaiaretic acid has been suggested to induce apoptosis through glutathione depletion, lipid peroxidation and mitochondrial depolarisation (Biswal et al., 2000; Tang and Honn, 1997). Mitochondrial membrane permeabilisation, resulting in release of several proteins such as cytochrome *c* from the intermembrane space, is supposed to play a crucial role in most pathways leading to apoptosis (Kroemer and Reed, 2000). The loss of mitochondrial potential and activation of caspase-3, a cysteine protease playing an important role in the process of apoptosis, have been reported to be related in a number of cell types (Kroemer and Reed, 2000; Tyas et al., 2000). In the present study, nordihydroguaiaretic acid induced mitochondrial depolarisation, cytochrome *c* release and caspase-3 activation in HL-60 cells. Our results confirm previously reported activation of caspase-3 during nordihydroguaiaretic acid-induced apoptosis, although caspases other than caspase-3 could also participate in this process (Biswal et al., 2000; Tyas et al., 2000). However, the effects of nordihydroguaiaretic acid are probably not related to its 5-lipoxygenase inhibiting properties. This conclusion is based on the finding that: (i) another inhibitor of leukotriene production, MK-886, did not induce apoptosis and it had no significant effect on TNF-α-induced apoptosis in HL-60 cells; (ii) undifferentiated HL-60 cells have negligible 5-lipoxygenase expression and activity (Bennett et al., 1993). We have confirmed this and found that neither indomethacin nor nordihydroguaiaretic acid induce 5-lipoxygenase expression in HL-60 cells. We have shown previously that micromolar concentrations of MK-886 significantly potentiate differentiation of HL-60 cells induced by various differentiation inducers, without a significant increase in apoptosis (Hofmanová et al., 1998), although several authors have suggested that MK-886 is a potent inducer of apoptosis in leukaemia cell lines (Datta et al., 1999; Dittmann et al., 1998). Our hypothesis is further supported by the finding that nordihydroguaiaretic acid induces apoptosis in the murine hematopoietic cell line FL5.12 without 5-lipoxygenase protein (Biswal et al., 2000). Thus, 5-lipoxygenase products of arachidonic acid metabolism are probably not involved in the effects of nordihydroguaiaretic acid on TNF-α-induced programmed cell death in our model, while other modes of action of nordihydroguaiaretic acid, such as glutathione depletion, may sensitise HL-60 cells to apoptosis induced by this cytokine.

Indomethacin, a widely used NSAID, induced cell death in HL-60 cells and it synergistically potentiated the effects of TNF-α. Recently, effects of cyclooxygenase inhibitors on apoptosis received considerable attention due to potential beneficial effects of NSAIDs in reducing the risk of colon cancer. However, their beneficial effects may be mediated through both cyclooxygenase-dependent and -independent pathways (Paik et al., 2000). It has been documented that high concentrations of indomethacin induce programmed cell death in K562 cells, as well as in chronic myeloid leukaemia cells (Zhang et al., 2000), although others have reported that indomethacin does not induce

apoptosis when used at cyclooxygenase-inhibiting concentrations (Bellosillo et al., 1998). In the present study, high concentrations of indomethacin were found to induce cell death in HL-60 cells; however, we did not observe the loss of mitochondrial potential and caspase-3 cleavage that were typical for nordihydroguaiaretic acid-induced apoptosis. Indeed, it has been reported that the mitochondrial depolarisation and cytochrome *c* release is not always necessary for induction of apoptosis in HL-60 cells (Finucane et al., 1999; Li et al., 2000). Other effector caspases could be involved in programmed cell death induced by indomethacin. The mechanism of indomethacin-induced apoptosis and of its potentiation of TNF-α-induced cell death could be cyclooxygenase-independent, as its concentrations that inhibit cyclooxygenase in vitro are lower than those inducing apoptosis (Bellosillo et al., 1998; Zhang et al., 2000). This is further supported by our finding that two other NSAIDs, piroxicam and ibuprofen (data not shown), had no effect on TNF-α-induced apoptosis. Interestingly, it has been documented that prostaglandins do not reverse the effects of NSAIDs on programmed cell death (Hanif et al., 1996). NSAIDs have been shown both to induce cyclooxygenase-2 expression and to inhibit mitogen-induced cyclooxygenase-2 expression (Paik et al., 2000). We found that indomethacin does not affect cyclooxygenase-2 expression in HL-60 cells. These results seem to support our conclusion that arachidonic acid-independent mechanisms are involved in the observed effects of indomethacin.

Indomethacin, as well as other NSAIDs, has been reported to bind and activate PPAR-γ and PPAR-α (Lehmann et al., 1997). It has also been documented that PPAR-γ is expressed in human myeloid leukaemia cells, including HL-60 cells (Asou et al., 1999). Recently, a mechanism involving induction of negative interference with NF-κB anti-apoptotic signalling pathway by PPAR ligands has been reported to play a role in the apoptosis of human macrophages (Chinetti et al., 1998). Therefore, we have hypothesised that high concentrations of indomethacin that were found to effectively potentiate programmed cell death induced by TNF-α could act through activation of PPAR-γ. However, we found that ciglitazone, a selective ligand of PPAR-γ, did not potentiate TNF-α-induced apoptosis. No effects were found also for Wy-14,643, a specific PPAR-α ligand. These results suggest that the effects of indomethacin were not mediated by a PPAR-dependent mechanism.

The exact relationship between cell maturation and apoptosis is unclear. It has been reported that myeloid cells may become resistant to various apoptotic stimuli, such as death ligands, irradiation and cytostatics, during the process of differentiation induced by *all-trans* retinoic acid. (Ketley et al., 1997; Kikuchi et al., 1996). Recently, we have found that *all-trans* retinoic acid rapidly induces resistance to TNF-α in HL-60 cells (Vondráček et al., 2001). In an attempt to further characterise the effects of indomethacin, we found that combination of TNF-α with indomethacin induce apoptosis in HL-60 cells treated with *all-trans* retinoic acid.

Another important physiological mediator of apoptosis is the Fas/APO-1/CD95 receptor, a surface receptor belonging to the TNF receptor family. Compared to TNF-α, relatively little is known about the role of arachidonic acid in apoptosis mediated by Fas. It has been reported that, unlike TNF-α, anti-Fas antibody does not induce cytosolic phospholipase A_2 activity (De Valck et al., 1998). While some authors have concluded that arachidonic acid or its metabolites do not play a significant role in Fas-mediated apoptosis (Cifone et al., 1995; Enari et al., 1996), others have suggested that lipoxygenase could play a critical role in FasL-induced apoptosis (Wagenknecht et al., 1997). It has been reported that type VI Ca^{2+}-independent phospholipase A_2 mediates fatty acid release in Fas-stimulated U937 cells and may play a modifying, although not essential, role in the apoptotic cell death process (Atsumi et al., 1998). In the present study, indomethacin treatment did not sensitise HL-60 cells to Fas-mediated programmed cell death.

In conclusion, indomethacin, a widely used NSAID, and nordihydroguaiaretic acid, a general lipoxygenase inhibitor, may significantly potentiate apoptosis induced by TNF-α in a myeloid leukaemia cell line. This interaction has a synergistic character. Furthermore, this effect can be observed in cells treated with *all-trans* retinoic acid, a differentiation inducer used for treatment of acute myeloid leukaemia patients, which induces resistance to TNF-α-induced apoptosis. However, the effects of indomethacin and nordihydroguaiaretic acid are probably not associated with the cyclooxygenase- and lipoxygenase-dependent activities of these compounds. The precise mechanisms of action of both compounds deserve further investigation. Nevertheless, our data raise the possibility that indomethacin could potentially be used to improve the treatment of human myeloid leukaemia.

Acknowledgements

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ORIGINAL ARTICLE

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Schedule-dependent synergism of taxol or taxotere with edatrexate against human breast cancer cells in vitro

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Abstract A new dihydrofolate reductase inhibitor, edatrexate (EDX), and the microtubule polymerization promotor, taxol (TXL) or taxotere (TXT), each have significant therapeutic activity against human breast cancer in clinical trials. Since they also have distinctly different mechanisms of actions and have mainly non-overlapping toxicities, they may be effective in combination in the treatment of this disorder. Schedule-dependent interactions between these taxanes and EDX against human breast adenocarcinoma cells (SK-Br-3) were quantitatively assessed in vitro to determine whether these interactions are synergistic or antagonistic. SK-Br-3 cells were grown as a monolayer in 96-well microplates. The dose-effect relationships of the drugs, singly and in combination, in inhibiting the growth over a 7-day period were determined by the SRB protein staining assays. Cell cultures were exposed to drug as a 3-h pulse at either 0–3 h or 24–27 h. Synergism or antagonism at different concentrations and at different effect levels were assessed with the medianeffect principle and the combination index-isobologram method using computer software. These methods were selected because they take into account both the potencies and the shape of the dose-effect curves. Exposure of cells to an equimolar combination of EDX + TXL (0–3 h) resulted in synergism at high effect levels. Pretreatment of cells with EDX (0–3 h) followed by TXL (24–27 h) showed even greater synergism in inhibiting cell growth. Moderate antagonism was observed with the reverse schedule. EDX + TXT (0–3 h) was additive, but pretreatment with EDX (0–3 hr) followed by TXT (24–27 h) showed synergism. However, the reverse order showed antagonism. Studies on another breast tumor cell line, ZR-57–1, also showed the schedule of EDX (0–3 h) + TXT or TXL (24–27 h) to be more synergistic than, the other two schedules examined. These results show potent schedule-dependent synergism of the combinations of TXL or TXT with EDX, and should form a rationale for designing clinical protocols utilizing these agents particularly for the treatment of breast cancer patients.

Key words Edatrexate · Taxol · Taxotere · Breast cancer · Combination treatment

Abbreviations *TXL* taxol · *TXT* taxotere · *EDX* 10-ethyl-10-deaza-aminopterin · *CI* combination index · *DRI* dose-reduction index

Introduction

The taxane derivatives, taxol (TXL) and taxotere (TXT), have been shown to exhibit extremely encouraging therapeutic activity against human malignancies, particularly refractory ovarian and breast cancers [1–7]. These agents are unique in that they enhance the polymerization of tubulin to stable microtubules by interacting stoichiometrically with microtubules in the absence of any cofactors [1, 3, 4]. Consequently, tumor cells are blocked in the mitotic phase of the cell cycle and are unable to replicate. As with other chemotherapeutic agents, the clinical utility of these taxane derivatives will depend upon their optimum use in combination with other clinically useful agents. Ideally, agents used in such combinations should exhibit minimal overlapping toxicities and have confirmed effects that reflect at least additive, if not, synergistic interactions in the target tumor cells.

Other studies [8] have shown that the classical folate analogue, edatrexate (EDX, 10-ethyl-10-deazaaminopterin)

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is similar to methotrexate (MTX) as an inhibitor of dihydrofolate reductase, but is more effectively transported and polyglutamylated in tumor cells. More importantly, there is greater accumulation of cytotoxic EDX polyglutamates [8] in tumor cells than in normal proliferative tissues that are sites of limiting toxicity. In other studies EDX has been shown to be more efficacious than MTX in vivo against murine ascitic and solid tumors and against human tumor xenografts that are resistant to MTX [9, 10]. EDX has been evaluated as a single agent in phase I and phase II studies [11, 12] and has been shown to have therapeutic activity against a number of tumors greater than MTX, particularly non-small cell lung cancer [13, 14] and breast cancer [15, 16]. Toward the further clinical development of EDX, combination therapeutic studies have shown that EDX administered with alkylating agents or cisplatin [17, 18] or vinca alkaloids [19] is highly effective against a number of MTX-resistant tumors. Retrospective analysis by the median-effect principle and combination index methodology [20–23] of the effect of the combination of EDX and cisplatin against human leukemia [24] and human lung cancer [25, 26] cells has shown that this combination is potently synergistic, which would suggest a basis for the high degree of effectiveness of, at least, the EDX plus cisplatin combination in vivo. The results of these studies have led to a number of clinical trials evaluating EDX [11, 26] in combination with these other agents.

The dose-limiting toxicities of taxane derivatives in patients are leukopenia and peripheral neuropathy [5–7]. In contrast, mucositis is the dose-limiting toxicity of EDX in patients [11–16]. Other toxicities including myelosuppression, small bowel disturbances, rash and pneumonitis, are generally mild to moderate in severity [11–16]. In view of the distinctly different mechanisms of action and the dissimilarity in toxicity associated with these clinically active agents, we initiated a prospective study of their combined effects against human breast cancer cells (SK-Br-3 and ZR-75-1) in cell culture. These studies used the median-effect principle and the combination index method [20–23], and computerized data analysis [27–28] for quantitation of synergism or antagonism of the agents. The results of these studies may have utility in designing combination chemotherapy clinical protocols for these two types of agents that have non-overlapping toxicities, particularly in breast cancer patients.

Materials and methods

Drugs and drug solutions

EDX, originally synthesized at SRI International, Menlo Park, Calif., was made available by the Ciba Geigy Corporation, Summit, N.J. EDX stock solutions were prepared at 2 mg/ml in 5% sodium bicarbonate and the pH was adjusted to 7.2. TXL was obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md., and was first dissolved in 50% ethanol and 50% cremophor and stored at −20°C. A stock solution was prepared (< 0.6 mg/ml TXL) prior to use by dilution with 0.5% ethanol, 5% cremophor and 90% saline. Further dilutions were made in MEM/f12 medium. TXT (RP56976) was obtained as a gift from Rhone-Poulenc Pharmaceuticals, France. TXT was first dissolved at 50 mg/ml in ethanol and stored at −20°C. Immediately prior to use, 1 vol of the ethanolic stock solution was mixed with 1 vol of polysorbate 80 and then 18 vol of 5% glucose in aqueous solution was added to make a 2.5 mg/ml stock solution. Further dilutions were made in MEM/f12 medium.

Cells and incubation

SK-Br-3 human breast adenocarcinoma and ZR-75-1 human breast carcinoma cells (obtained from American Type Culture Collection, Rockville, Md.) were used. They were grown as a monolayer in 96-well plates, in MEM/f12 supplemented with 10% fetal bovine serum, under an atmosphere containing 5% CO_2 in an incubator at 37°C. A suspension of 8×10^3 cells in 100 µl was added to each experimental well 24 h before drugs were added. Plates were read when control wells were almost confluent (7 days) using the SRB protein staining assay [29].

Drug combinations and schedules

EDX combined with either TXL or TXT was explored with three different pulse treatment schedules: (a) simultaneous exposure: EDX, 0–3 h + another drug, 0–3 h; (b) sequential exposure: EDX, 0–3 h + another drug, 24–27 h; and (c) reverse exposure: another drug, 0–3 h + EDX, 24–27 h. For each drug combination, three 96-well plates were used, one for each pulse schedule. Each experiment was repeated several times as indicated in Tables 1–3.

Each plate included wells with the drugs combined, single-drug controls, solvent controls and cell controls. All single-drug wells as well as the control wells were handled in exactly the same way as the combination wells except for the concentration and timing of drug additions. Drugs were serially diluted twofold across the wells and any rows not getting drug at any time had medium passed from well to well. Similarly, when drugs were removed at the end of the pulse treatment (using a sterile pasteur pipette attached to vacuum) the medium of all control wells was similarly emptied and all were given fresh medium. Plates were read when control wells were almost confluent (7 days). The SRB protein staining assay [29] was used and absorbance was read on a microplate reader (Bio-Tek, model EL-340). Fractional inhibition (f_a) of cell growth (percent inhibition/100) as compared to untreated cell controls at the end of inhibition measurement was calculated.

Quantitation of synergism or antagonism

For each of the individual drugs (EDX, TXL, TXT) and their two drug combinations, the dose-effect relationships obtained from two fold serial dilution and the SRB cytoxicity assay were subjected to a median-effect plot analysis [23, 28]. Briefly, the median-effect equation is expressed as:

$$(f_a)/(f_u) = (D/D_m)^m$$

or

$$\log (f_a/f_u) = m \log D - m \log D_m \qquad \text{Eq. 1}$$

For EDX, D is the dose of EDX, D_m is the median-effect dose (ED_{50} or IC_{50}) of EDX, f_a is the fractional inhibition (e.g. $f_a = 0.95$ for 95%

Table 1 Dose-effect relationship parameters of EDX, TXL and TXT against the growth of SK-Br-3 and ZR-75–1 breast adenocarcinoma cells with different drug exposure times during a 7-day incubation period as described in Materials and methods. (m, D_m, and r are the slope, antilog of the x-intercept, and the linear correlation coefficient of the median-effect plot [Eq. 2–23] which signify the shape of the dose-effect curve, the potency (IC_{50}) and the conformity of the data to the mass-action law, respectively [27]; n is the number of sets of dose-effect relationship experiments carried out). Values are mean ± SEM for SK-Br-3 cells, and mean ± variation for ZR-75-1 cells

Drug	Breast cancer cells	D_m (μM)	m	r	n
EDX (0–3 h)	SK-Br-3	0.085 ± 0.022	0.802 ± 0.173	0.851 ± 0.050	11
	ZR-75-1	0.273 ± 0.165	0.811 ± 0.281	0.880 ± 0.010	2
TXL (0–3 h)	SK-Br-3	0.039 ± 0.020	0.648 ± 0.105	0.943 ± 0.013	8
	ZR-75-1	0.020 ± 0.004	0.738 ± 0.157	0.919 ± 0.044	2
TXT (0–3 h)	SK-Br-3	0.013 ± 0.004	1.039 ± 0.172	0.955 ± 0.017	5
	ZR-75-1	0.043 ± 0.012	0.541 ± 0.111	0.875 ± 0.081	2
EDX (24–27 h)	SK-Br-3	0.120 ± 0.043	0.964 ± 0.350	0.925 ± 0.020	6
	ZR-75-1	0.435 ± 0.193	0.595 ± 0.046	0.853 ± 0.013	2
TXL (24–27 h)	SK-Br-3	0.071 ± 0.027	0.734 ± 0.072	0.971 ± 0.014	4
	ZR-75-1	0.047 ± 0.017	0.785 ± 0.359	0.960 ± 0.001	2
TXT (24–27 h)	SK-Br-3	0.017 ± 0.006	1.248 ± 0.297	0.905 ± 0.053	4
	ZR-75-1	0.049 ± 0.026	0.726 ± 0.299	0.886 ± 0.059	2

inhibition by EDX), f_u is the fraction unaffected ($1 - f_a$), and m is the coefficient signifying the slope of the dose-effect curve for EDX ($m = 1$, > 1, and < 1 indicate a hyperbolic, sigmoidal, and negatively sigmoidal curve, respectively). Based on Eq. 1, the median-effect plot of $x = \log D$ vs $y = \log[f_a/(1 - f_a)]$ yields a straight line with a slope (m) and an x-intercept of $\log D_m$. Thus, the m and D_m parameters of EDX can be easily determined quantitatively (Table 1). The same procedure was carried out for TXL, TXT and the combinations. The conformity of the experimental data to the median-effect principle of the mass-action law is indicated by the linear correlation coefficient (r) of the median-effect plot (Table 1).

From the m and D_m values, the isoeffective dose (D_x) for any effect level (e.g. ED_{70} for $f_a = 0.7$, ED_{90} for $f_a = 0.9$) for each drug or for the combinations can be easily determined by rearrangement of Eq. 1.

$$D_x = D_m[f_a/(1 - f_a)]^{1/m} \qquad \text{Eq. 2}$$

Synergism or antagonism for EDX plus TXL or TXT is determined by using m and D_m parameters and Eq. 2 by substituting D_x values into the combination index (CI) equation of Chou and Talalay [21–23], where $CI < 1$, > 1 and > 1 indicate synergism, an additive effect, and antagonism, respectively. Based on the classical isobologram for mutually exclusive drug effects relative to the end-point of measurement, the CI value for x% inhibition is calculated as:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \qquad \text{Eq. 3}$$

For example, at 90% inhibition level, $(D_x)_1$ and $(D_x)_2$ are the doses for 90% inhibition by EDX and TXL, respectively, which can be obtained from Eq. 2; and $(D)_1$ and $(D)_2$ in the numerators are the doses of EDX and TXL in combination that also inhibit cell growth by 90% (i.e. isoeffective compared with the single drugs alone). If the calculation is based on the conservative isobologram equation, assuming that the effects of two drugs are mutually nonexclusive (i.e totally independent relative to the end-point of measurement), then an approximated third term designated as the product of the first two terms should be added to Eq. 3. For simplicity, CI values obtained from the classical isobologram equation (Eq. 3) have frequently been used, but the underlying assumption needs to be stated. When exclusivity is unknown, the CI value is routinely calculated in both ways [27, 28].

The dose-reduction index (DRI) defines the extent of dose reduction possible in a combination for a given degree of effect as compared with the dose of each drug alone [23, 28]: $(DRI)_1 = (D_x)_1/(D)_1$ and $(DRI)_2 = (D_x)_2/(D)_2$; therefore, the relationship between DRI and CI is expressed as

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2} \qquad \text{Eq. 4}$$

Computer softwares [27, 28] can be used for fully automated data analysis based on Eqs. 1–4. Example calculations on a set of crude data, using a pocket calculator, are demonstrated in references 22 and 24.

Results

Single-drug efficacy and parameters

As shown in Table 1, SK-Br-3 and ZR-75–1 breast cancer cells showed characteristic differences in their sensitivity to drugs (D_m values or IC_{50} values) and the shapes of the dose-effect curves (m values) at both 0–3 h and 24–27 h drug exposure. EDX and TXT were about threefold more effective against SK-Br-3 cells than against ZR-75–1 cells. By contrast TXL was about twofold more effective against ZR-75–1 cells than against SK-Br-3 cells. In addition, ZR-75–1 cells showed shallower dose-effect curves (lower m values) toward TXT and EDX, and somewhat steeper dose-effect curves (higher or similar m values) for TXL, when compared with SK-Br-3 cells.

Drug combination and scheduling studies

To avoid inter-experimental variations, each determination of synergism/antagonism was carried out in a large-scale experiment using a 96-well microplate reader so that the parameters (D_m, m and r) and the CI values were calculated from the dose-effect relationships within each experiment. The experiments were repeated four or more times and the average CI and its

Table 2 Combination effects of EDX + T[redacted]uring a 7-day incubation against SK-Br-3 and ZR-75-1 cell growth. CI < 1, = 1, and > 1 indicate synergism, an additive effect, and antagonism, respectively. Values are mean ± SEM for SK-Br-3 cells and mean ± variation for ZR-75-1 cells. Each data set for the CI calculations consisted of dose-effect relationships of two single drugs and their combinations obtained from individual experiments (*n* number of sets of dose-effect relationship exper[redacted]ts). Each dose-effect relationship consisted of six to eight drug concentrations in duplicate. D_m and *m* values were used for calculating the CI values based on the CI equation of Chou and Talalay [21–23] using computer software [27]. Equations used were: $D_x = D_m\ [f_a/(1-f_a)]^{1/m}$ and $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$, where D_x is the dose (concentration) for *x*% inhibition

Drug Combination (1:1 molar ratio)	Breast cancer cells	CI value at IC_{50}	IC_{75}	IC_{90}	IC_{95}	*n*
EDX + TXL	SK-Br-3	3.324 ± 1.596	1.105 ± 0.154	0.740 ± 0.160	0.595 ± 0.167	4
(0–3 h) (0–3 h)	ZR-75-1	1.644 ± 0.018	2.916 ± 1.551	6.791 ± 5.65	13.009 ± 12.00	2
EDX + TXL	SK-Br-3	1.335 ± 0.289	0.684 ± 0.160	0.486 ± 0.173	0.446 ± 0.174	5
(0–3 h) (24–27 h)	ZR-75-1	0.767 ± 0.133	0.693 ± 0.202	0.646 ± 0.251	0.621 ± 0.276	2
EDX + TXL	SK-Br-3	1.088 ± 0.223	0.854 ± 0.145	1.004 ± 0.280	1.714 ± 0.822	5
(24–27 h) (0–3 h)	ZR-75-1	0.507 ± 0.373	0.714 ± 0.032	2.160 ± 1.610	6.182 ± 5.690	2

Table 3 Combination effects of EDX + TXT during a 7-day incubation against SK-Br-3 and ZR-75-1 cell growth. For an explanation of the CI values and their calculation, see Table 2

Drug Combination (10:1 molar ratio)	Breast cancer cells	CI values at IC_{50}	IC_{75}	IC_{90}	IC_{95}	*n*
EDX + TXT	SK-Br-3	1.447 ± 0.174	1.457 ± 0.255	1.497 ± 0.330	1.540 ± 0.381	3
(0–3 h) (0–3 h)	ZR-75-1	1.181 ± 0.146	0.783 ± 0.092	0.611 ± 0.006	0.552 ± 0.059	2
EDX + TXT	SK-Br-3	1.233 ± 0.098	1.050 ± 0.056	0.900 ± 0.060	0.807 ± 0.070	3
0–3 h) (24–27 h)	ZR-75-1	0.865 ± 0.131	0.661 ± 0.129	0.509 ± 0.118	0.428 ± 0.108	2
EDX + TXT	SK-Br-3	1.240 ± 0.040	1.278 ± 0.027	1.495 ± 0.135	1.680 ± 0.260	3
(24–27 h) (0–3 h)	ZR-75-1	0.813 ± 0.304	1.639 ± 0.002	5.473 ± 2.845	15.48 ± 11.62	2

variations were then determined for EDX + TXL schedules (Table 2) and EDX + TXT schedules (Table 3). Since the experiments involved pulse drug exposure which includes washing of cells with fresh medium, this manipulation of cells would increase the variability of the data when compared with experiments involving continuous drug exposure without a washing, as has been described previously for EDX and cisplatin combinations [25].

Among the three treatment schedules for EDX + TXL given in Table 2, EDX (0–3 h) + TXL (24–27 h) in both SK-Br-3 and ZR-75-1 cells showed a greater synergism than the (0–3 h) simultaneous exposure schedule or the reverse schedule with EDX (24–27 h). For the EDX (24–27 h) + TXL (0–3 h) schedule, antagonism was observed for both SK-Br-3 and ZR-75-1 cell lines. The EDX (0–3 h) + TXL (0–3 h) simultaneous schedule showed mixed results with SK-Br-3 cells showing antagonism at low effect levels and synergism at high effect levels, and ZR-75-1 cells showing antagonism.

As shown in Table 3, among the three schedules for EDX + TXT, EDX (0–3 h) + TXT (24–27 h) was consistently synergistic in both SK-Br-3 and ZR-75-1 cells, EDX (0–3 h) + TXT (0–3 h) showed mixed results, and EDX (24–27 h) + TXT (0–3 h) showed various degrees of antagonism.

A set of data for an EDX (0–3 h) + TXL (24–27 h) experiment were used to illustrate the actual data analysis using the computerized simulation. For the f_a–CI plot (Fig. 1), the open circles are the actual experimental data points and the curve was obtained from computer simulation using Eqs. 2 and 3. In order to condense the *y*-axis for all CI values, Fig. 1 was plotted in F_a-log(CI) format. This format made the CI scale symmetrical at log(CI = 1) = 0. The results indicate that synergism (i.e. CI < 1) occured at high f_a values. It should be noted that in actual cancer chemotherapy, $f_a > 0.95$ or $f_a > 0.99$ is considered to be more relevant to actual practice. Therefore, synergism at high effect levels in these experiments is more important than that at low effect levels.

Figure 1 is the effect-oriented plot (i.e. f_a-CI plot) for indicating synergism or antagonism. The same set of data can be presented in a dose-oriented format [21, 28] as shown in Fig. 2. Figure 2 is the computer-generated isobologram at ED_{50}, ED_{70} and ED_{90} effect levels for both EDX and TXL. Combination data points that fall to the lower left of the diagonal line of each effect level indicate synergism, those that fall on the diagonal line indicate an additive effect, and those that fall to the upper right indicate antagonism [21, 23]. In Fig. 2, the combination effect at ED_{50}, ED_{70}, and ED_{90} shows an additive effect, synergism

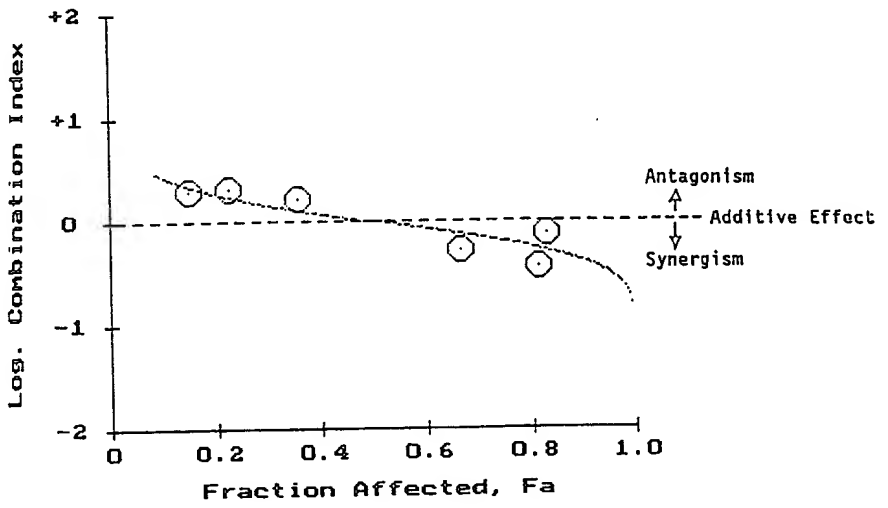

Fig. 1. Computer-simulated CI at various effect levels (f_a) for the combination of EDX (0–3 h) with TXL (24–27 h) at an equimolar ratio. The experiment was carried out on SK-Br-3 cells. Since CI < 1, $= 1$, and > 1 indicate synergism, an additive effect, and antagonism, respectively, in the f_a–log(CI) plot, log(CI) < 0, $= 0$, and > 0 indicate synergism, an additive effect, and antagonism, respectively. *Open circles* are the actual combination data points, and the curve was obtained from computer simulation

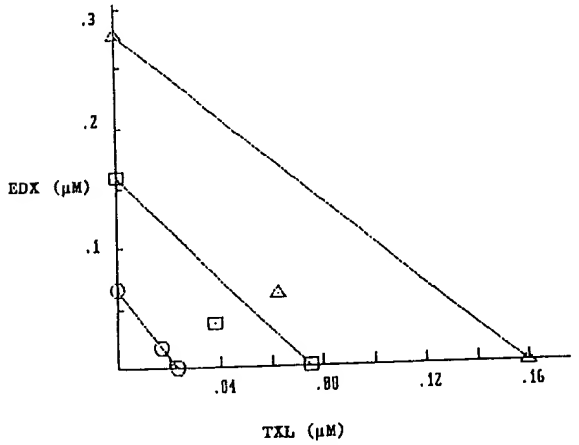

Fig. 2. The computer-generated classical isobologram for the combination of EDX (0–3 h) and TXL (24–27 h) at ED_{50} (○), ED_{70} (□) and ED_{90} (△) dose levels. The same data as those in Fig. 1 were used for the analysis. *x*- and *y*-axes show the ED_{50}, ED_{70} and ED_{90} values for taxol and EDX, respectively. The combination data points that fall on the diagonal line, to the lower left and to the upper right, represent an additive effect, synergism, and antagonism, respectively. Note that the ED_{50} isobologram shows an additive effect, whereas the ED_{70} and ED_{90} isobolograms show synergism

and synergism, respectively. Isobolograms at any other effect levels for EDX and TXL (e.g. ED_{95} or ED_{99} isobologram) can be readily constructed using computer software [27, 28]. The method for step-by-step calculation with a pocket calculator has been demonstrated previously [23, 24]. Figures 1 and 2 should give numerically identical conclusions since they are based on the same set of data and have used the same CI equation of Chou and Talalay [21–23]. It should be noted that isobolograms in Fig. 2 cannot show too many effect levels since they tend to be congested and become difficult to read whereas F_a–CI or F_a–log(CI) plots do not have such a restriction (Fig. 1).

Discussion

The combination of TXL or TXT with EDX would appear to offer considerable potential in the treatment of breast and other human cancers that are responsive to these agents. In view of the non-overlapping toxicities associated with these agents [6, 12], the tolerance of patients to these combinations is a reasonable expectation. Moreover, the present studies showed synergism between both taxanes and EDX in the context of the two breast tumor cell culture systems. The synergism observed appeared to be schedule-dependent. There were mixed results of moderate synergism or antagonism following simultaneous exposure of SK-Br-3 or ZR-57-1 cells to taxane and EDX at 0–3 h, but synergism was consistently observed when EDX was given 24 h before either taxane. Most importantly, the synergism seen with those schedules was greatest at high-effect levels, a situation most relevant to the clinical use of these agents. It is not uncommon that drug combinations tend to be antagonistic at low doses and synergistic at high doses [21–24]. Interesting, as well, were other results showing that sequential administration of these agents in the reverse order was antagonistic. The causes for the schedule dependence of these effects is unknown. We also noted that the synergism attained for the combination of TXL with EDX was greater than for TXT with EDX, while TXT was threefold more cytotoxic than TXL. However, this result may only reflect the cytotoxic properties of these agents against these two particular cell lines and not necessarily others, and no general conclusion is offered in this regard.

These results may have particular relevance to the treatment of metastatic breast cancer. Standard combination therapy for this disorder, although achieving relatively high response rates (reviewed in reference 16), has had little impact on survival of these patients.

Although doxorubicin is the most active single agent in this disorder, its use is limited by acute toxicities and irreversible cardiotoxicity with high cumulative doses. TXL, TXT and EDX are among the new promising antitumor agents that are under active investigation in breast cancer and have shown promise as single agents against metastatic diseases [15, 16, 30]. The administration of recombinant human granulocyte colony stimulating factor reduces the incidence, depth and duration of neutropenia induced by TXL [31, 32] compared with previously reported experience. These findings, and others pertaining to the non-overlapping toxicities of these agents discussed above, prompted our investigation in vitro as to whether these two categories of agents might show a synergistic dose reduction in their effects on human breast cancer cells in the absence of granulocyte colony stimulating factor. These results have prompted the initiation of phase I clinical trials of TXL with EDX and planning for upcoming phase II trials at our institution and elsewhere.

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Synergistic Effect of CGS16949A and 5-Fluorouracil on a Human Breast Cancer Cell Line

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Key Words

CGS16949A · 5-Fluorouracil · Breast cancer · SK-BR-3 cell line

Abstract

The effects of the aromatase inhibitor, CGS16949A, and the fluoropyrimidine, 5-fluorouracil (5-FU), on cell cycle distribution and growth were studied using FACS analysis and MTT assay in the human breast cancer cell line, SK-BR-3. CGS16949A induced an increase in the G0–G1 fraction on SK-BR-3 cells, and the growth inhibition rate of the combination of both (65.7 ± 3.0%) was significantly higher than 10 n*M* CGS16949A (37.9 ± 6.9%) or 100 μg/ml 5-FU (45.6 ± 4.5%); $p < 0.01$). Administering 5-FU after preincubation with CGS16949A significantly increased the combined cytotoxic efficacy, suggesting that clinical therapy using this combined therapy may be more efficient.

Introduction

Inhibition of estrogenic stimulation is the main target in endocrine treatment of breast cancer. This is achieved either by blocking the estrogen receptor or by the inhibition of estrogenic production. Aromatase is present in human breast tumors and in breast cancer cell lines suggesting the possibility of in situ estrogen production via the androstendione to estrogen and estradiol pathway [1–3]. CGS16949A is a nonsteroidal competitive inhibitor of this enzyme which plays an important role in the blocking of estrogen synthesis. CGS16949A has significant antitumor activity in the treatment of breast cancer [4–6], and has been reported to be a very potent and highly selective inhibitor of the aromatase enzyme system compared with the effect of aminogluthetimide, a clinically used first-generation aromatase inhibitor [7]. Regarding the effect of the aromatase inhibitor on cell kinetics, aminogluthetimide has been found to induced an increase in the G0–G1 fraction and a decrease in the G2+M fraction in vivo [8]. However, to our knowledge, the direct effect of CGS16949A on carcinoma cell cycle kinetics in vitro has not been reported. On the other hand, 5-fluorouracil (5-FU) is one of the cytotoxic agents which interferes with DNA synthesis and has been widely used as adjuvant chemotherapy in women with advanced breast cancer with or without estrogen receptor. We have studied the cytokinetic and cytotoxic effect of CGS16949A and the antitumor effect of the combination of CGS16949A with 5-FU against a human breast cancer cell line in vitro.

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Methods

Cell Line and Cell Culture

The human breast cancer cell line, SK-BR-3, was purchased from the American Type Culture Collection (Rockville, Md., USA). This cell line was grown in 25-cm^2 tissue culture flasks with McCoy's 5a medium (Gibco BRL, N.Y., USA) containing 10% fetal calf serum (FCS). SK-BR-3 was incubated at 37°C in air with 5% CO_2, and the media were changed every 3rd day. Estrogen receptor was showed in SK-BR-3 cells [9].

Chemicals

CGS16949A was provided by Novartis Pharma (Basel, Switzerland) and 5-FU was purchased from Kyowa Hakko Co., Ltd. (Tokyo, Japan) in an injectable form. Propidium iodode (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

Aromatase Activity Assay

Aromatase activity was measured using the tritiated water method as previously described [10]. SK-BR-3 cells reached 80% of confluence, medium was changed to FCS free medium and the cells were pre-incubated for 48 h. The medium was changed and cells were incubated with [1β-^{3}H]-androstenedione (20 n*M*, 24.7 Ci/mmol) for additional 5 h. In some dishes, an aromatase inhibitor, CGS16949A, was added simultaneously. The reaction was terminated by the addition of 0.5 ml trichloroacetic acid (10%). The medium was transferred to a test tube containing with 1.5 ml charcoal (30%) to remove residual steroids, and the mixture was incubated at 37°C in water for 30 min. The mixture was centrifuged (3,000 *g*) for 10 min and the supernatant was filtered. The radioactivity of [^{3}H]-water was measured in a scintillation counter (Aloka LSC-651, Tokyo, Japan).

Flow Cytometry

10 n*M* CGS16949A-untreated or treated cells were seeded in triplicate at a density of 5 × 10^5 cells/dish in 10 ml feeding medium in 60-mm Petri dishes. Cells reached 80% of confluence, and were harvested by trypsinization, washed with PBS, and then fixed in ice-cold 70% ethanol and treated with 100 μg/ml of RNase A (Sigma) for 15–30 min. After resuspending in the PI solution with a concentration of 100 μg/ml, cells were subjected to fluorescence-activated cell-sorter (FACS) analysis. Cell cycle profiles and distributions were determined by flow cytometry, FACS scan (Becton Dickinson, Oxnard, Calif., USA).

Growth Inhibition

The antiproliferative effect of CGS16949A on the in vitro growth profile of SK-BR-3 cells was examined with MTT assay using the method of Mosmann [11]. Subconfluent culture of SK-BR-3 cells was trypsinized and washed twice with the medium. Single cell suspensions with 20% FCS were seeded at a density of 1 × 10^4 cells/well in 50 μl nutrient medium. CGS16949A and 5-FU dissolved in the medium were added at twice the final concentration at a volume of 50 μl/well in quadruplicate wells. After incubation for 72 h, 10 μl of MTT solution and succinic acid were added to each well. The inhibition rate was calculated as follows:

$$\text{Inhibition rate} = [(c - t)/(c - m)] \times 100\,(\%),$$

where c = non-treatment absorbance units; m = background absorbance units, and t = treatment absorbance units. This experiment was independently performed three times.

Treatment groups were divided into: CGS16949A alone, 5-FU alone, and CGS16949A and 5-FU combined. The CGS16949A alone group was composed of 2 dose groups, 10 and 100 nmol/ml, and the 5-FU alone group had 3 dose groups, 10, 100 and 1,000 μg/ml. The combined CGS16949A and 5-FU group was composed of 3 groups for the administration of 10 n*M* CGS16949A and 100 μg/ml 5-FU: simultaneous administration of CGS16949A and 5-FU (group 1); CGS16949A administration after 24-hour treatment with 5-FU (group 2), and 5-FU administration after 24-hour treatment with CGS16949A (group 3).

Statistical Analysis

The one-factor ANOVA test was used to examine the statistical significance of change in aromatase activity and inhibition rates.

Results

Effect of CGS16949A on Aromatase Activity in SK-BR-3

The aromatase activity of SK-BR-3 cells without CGS16949A was 3,250 fmol/h/mg protein. When 1 n*M* CGS16949A was administered, the activity became reduced, corresponding to 60% of that without CGS16949A. Similarly, when 10 and 100 n*M* of CGS16949A were administered, the activities showed 18.7 ± 5.8 and 2.7 ± 0.3% of that without CGS16949A, respectively. Aromatase activity was significantly inhibited ($p < 0.01$) by the addition of CGS16949A in a concentration-dependent manner (fig. 1).

Effect of CGS16949A for Cell Cycle

The capacity of CGS16949A to inhibit cell cycle progression was evaluated via flow cytometry. A representative example depicting the effect of CGS16949A treatment on cell cycle phase distribution in the SK-BR-3 cell line is given in figure 2. Treatment with 10 n*M* CGS16949A for 48 h resulted in the accumulation of cells in the G0–G1 phase (62.0 ± 1.1%) compared with that at the initial time (55.0 ± 4.6%). The change in S-phase accumulation was slightly decreased in the cells treated for 48 h, and soon recovered in those treated for 72 h (37.0 ± 2.6 and 41.5 ± 0.7%, respectively). A concurrent decrease in the percentage of cells in G2+M phase was observed in cells treated with CGS16949A (1.3 ± 1.9%) for 48 h compared with those at the initial time (4.0 ± 3.0%). The G2+M phase depletion was more pronounced in cells treated with CGS16949A for 72 h (0.5 ± 0.7%). The percentage of cells in the cell cycle returned to the initial percentage after treatment for 96 h.

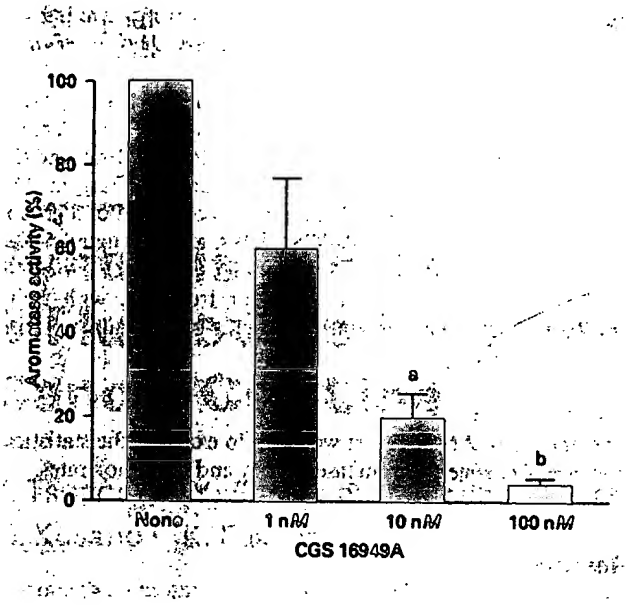

Fig. 1. Effect of aromatase inhibitor CGS16949A on aromatase activity in SK-BR-3 cells as measured with the tritiated water-release method. Bars represent mean ± SEM from four determinations in two independent experiments. [a] $p < 0.01$ compared with 1 n*M* CGS16949A; [b] $p < 0.01$ compared with 10 n*M* CGS16949A.

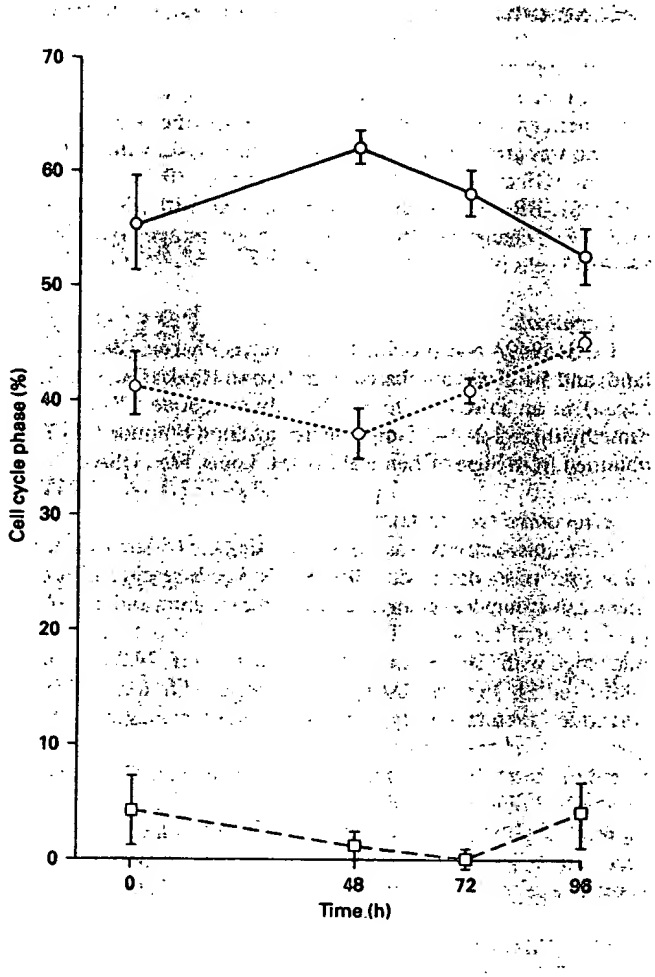

Fig. 2. Cell cycle distributions of SK-BR-3 cells were determined by flow cytometry. 10 n*M* CGS16949A reduced the S (○) and G2+M (□) fractions and increased the G0–G1 (○) fraction of SK-BR-3 cells growing in medium. Data are expressed as mean ± SEM from four determinations in two independent experiments.

Chemosensitivity Analysis

Inhibition rates of the 10- and 100-n*M* concentrations in the CGS16949A groups were estimated at 37.9 ± 6.9 and 29.5 ± 6.9% (fig. 3a). Those of 5-FU alone increased in a dose-dependent manner, and were 31.5 ± 17.1% for 10 μg/ml; 45.6 ± 4.5% for 100 μg/ml, and 66.0 ± 5.7% for 1,000 μg/ml. A statistical difference was seen between 10 and 1,000 μg/ml, and 100 and 1,000 μg/ml ($p < 0.01$; fig. 3b).

The inhibition rates of 10 n*M* CGS16949A alone, 100 μg/ml 5-FU alone, and the combination of both were 37.9 ± 6.9, 45.6 ± 4.5, and 65.7 ± 3.0%, respectively. There was a significant difference in the inhibition rate between the combination of both and 10 n*M* CGS16949A alone or 100 μg/ml 5-FU alone ($p < 0.01$; fig. 4). The combination of both showed the almost same inhibition rate as 1,000 μg/ml of 5-FU alone.

Regarding the combination groups, the inhibition rates in groups 1, 2, and 3 were 65.7 ± 3.0, 60.5 ± 2.5, and 34.5 ± 2.1%, respectively. A statistical difference was seen between groups 1 and 3, and groups 2 and 3 ($p < 0.01$, respectively; fig. 5). The mean inhibition rate was more than 60% in groups 1 and 2 and this was similar to the effect for the administration of 1,000 μg/ml 5-FU alone. Group 3 did not effectively inhibit the SK-BR-3 cells and its inhibition rate was almost the same as that evaluated by the administration of 10 μg/ml 5-FU.

Discussion

The mechanism by which aromatase inhibitors modulate breast cancer growth has been known to block estrogen synthesis via androstenedione to estrogen [12], but the cell cycle kinetic effect of CGS16949A in a cultured human breast cancer cell line has not been defined. In the present study, the aromatase activity of SK-BR-3 cells was

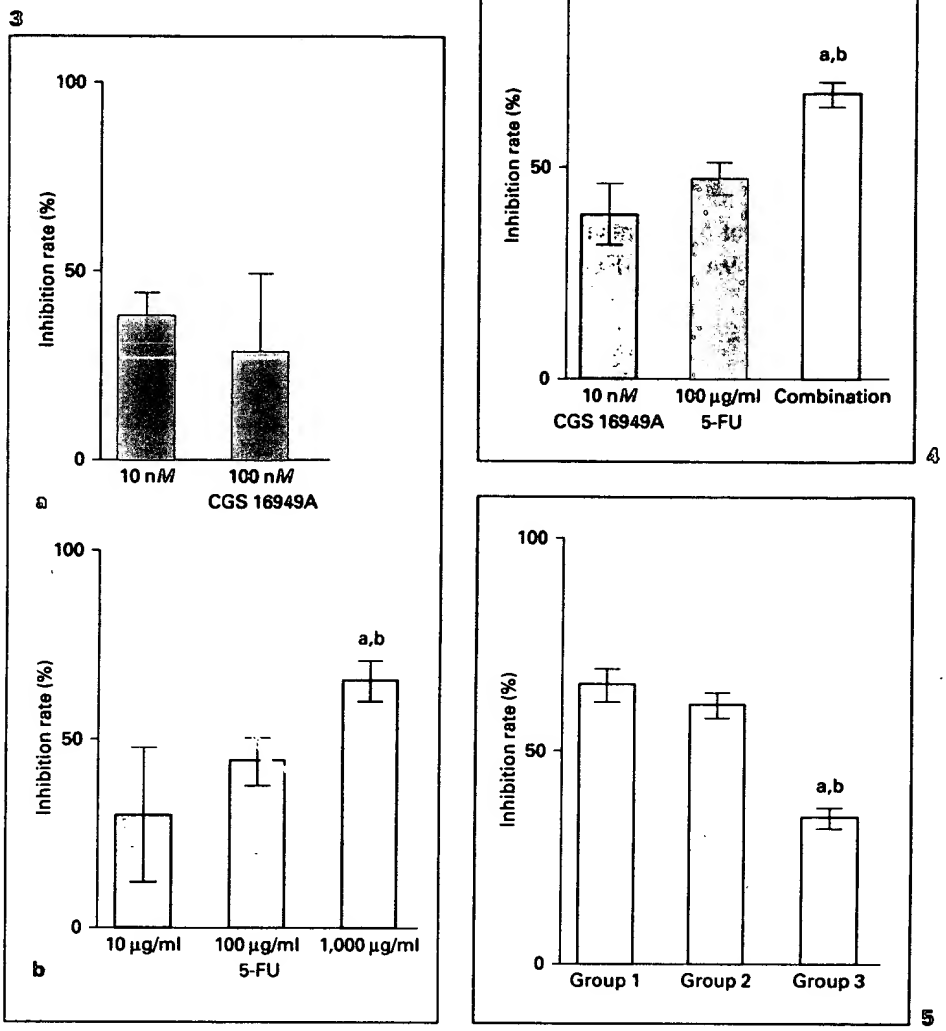

Fig. 3. Effect of aromatase inhibitor CGS16949A (a) and 5-FU (b) on cell proliferation in SK-BR-3 cells as measured with MTT assay. Columns = Means of three experiments in quadruplicate per group; bars = SD. $^{a}p < 0.01$ compared with 10 µg/ml 5-FU; $^{b}p < 0.01$ compared with 100 µg/ml 5-FU.

Fig. 4. Effect on cell proliferation of 10 n*M* CGS16949A and 100 µg/ml 5-FU, given alone or in combination in SK-BR-3 cells, as measured with the MTT assay. Columns = Means of three experiments in quadruplicate per group; bars = SD. $^{a}p < 0.01$ compared with 10 n*M* CGS16949A; $^{b}p < 0.01$ compared with 100 µg/ml 5-FU.

Fig. 5. Inhibition rate as a result of the MTT assay evaluating the inhibitory effect on the proliferation of SK-BR-3 cells in three groups: group 1 = simultaneous administration of CGS16949A and 5-FU; group 2 = CGS16949A administration after 24-hour treatment with 5-FU, and group 3 = 5-FU administration after 24-hour treatment with CGS16949A. Columns = Means of three experiments in quadruplicate per group; bars = SD. $^{a}p < 0.01$ compared with group 1; $^{b}p < 0.01$ compared with group 2.

3,250 fmol/h/mg protein using the tritiated water-release method, and was decreased in a dose-dependent manner. The cell cycle distribution of SK-BR-3 cells was determined by flow cytometry. 10 n*M* CGS16949A reduced the S and G2+M fractions and induced the G0–G1 fraction of SK-BR-3 cells growing in vitro. de Launoit and Kiss [8] characterized the in vivo influence of aminoglutethimide as an aromatase inhibitor on the percentage of cell cycle kinetic parameters in the G0–G1, S and G2+M phases of the mouse MTX mammary tumor. The results of in vitro and in vivo studies have proved that the ability to synchronize the endocrine-sensitive cells in the G0–G1 phase with antiaromatase therapy could be used to design more rational treatment strategies.

5-FU is an S-phase-specific agent and has two actions on the cell cycle according to the drug doses [13]. The high dose of 5-FU had a cytotoxic action, and the low doses of 5-FU disturbed cell cycle progression but did not affect cell survival. In the present study, the low dose of 5-FU (10 µg/ml) showed the same antitumor effect as CGS16949A, and its mean inhibition rate was under 40%, whereas this was more than 60% when the high dose of 5-FU (1,000 µg/ml) was administered. The antitumor effects of the combination therapy were altered according to the administration time of CGS16949A. CGS16949A treatment after the addition of 5-FU had a synergistic effect, while CGS16949A treatment before the addition of 5-FU did not effectively inhibit the proliferation of SK-

BR-3 cells. 5-FU interfered with DNA synthesis, and cells not in the S phase might be spared from their cytotoxic action. CGS16949A had phase-limiting cytotoxic activity because blocking effects on the progression of G1 cells into S phase have been observed. The synergistic antitumor effect of the simultaneous administration of 10 n*M* CGS16949A and 100 μg/ml 5-FU was proved, and the inhibition rate was higher than that of 100 μg/ml 5-FU alone and the same as that of 1,000 μg/ml 5-FU. The effective dose of 5-FU was reduced by combined therapy with CGS16949A and 5-FU.

In conclusion, it is necessary to understand the effect of the antitumor agents on cell cycle progression, because it is essential in designing effective chemoendocrine therapy regimens. Our results have potentially important implications in the treatment of patients with breast cancer, especially concerning the use of aromatase inhibitors combined with 5-FU. The combination of CGS16949A and 5-FU was often used regardless of hormone receptor status as adjuvant therapy. This study suggests that the chemoendocrine therapy with CGS16949A and 5-FU may allow the effective dose of 5-FU to be reduced.

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Sequence Dependence of Alimta™ (LY231514, MTA) Combined with Doxorubicin in ZR-75-1 Human Breast Carcinoma Cells

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Abstract. *Alimta™ is a new-generation antifolate with inhibitory activity against multiple enzymes, including thymidylate synthase, glycinamide ribonucleotide formyltransferase and dihydrofolate reductase. Alimta is undergoing broad phase II evaluation as a single agent, and preliminary results show responses in several tumor types, including breast carcinoma. Doxorubicin is often used in combination chemotherapy of breast cancer. Because the two drugs have mechanisms of action that might be complementary, we investigated a possible synergism between doxorubicin and Alimta on growth inhibition of ZR-75-1 human breast carcinoma cells. Cytostatic activity was evaluated using semi-automated MTT assays, and drug interactions were determined using CalcuSyn (Chou/Hayball) multiple drug effect analyses. The cells were exposed to Alimta or doxorubicin as single agents and combinations for 24 hours starting at the time of plating or for 72 hours starting 24 hours after plating with a total culture time of 96 hours. Preincubation with Alimta for 24 hours followed by exposure to doxorubicin for 72 hours resulted in highly synergistic activity, whereas the opposite sequence or simultaneous exposure produced mainly an additive response. DNA flow cytometry studies indicated that Alimta causes a build-up of cells near the G1/S interface after 24 hours of incubation. The data suggest that, to obtain maximal antitumor activity, Alimta should precede doxorubicin when the drugs are given in combination chemotherapy protocols.*

Alimta is a structurally novel antifolate antimetabolite that possesses the unique 6-5- fused pyrrolo[2,3-d]pyrimidine nucleus (1) instead of the more common 6-6-fused pteridine or quinazoline ring structure. The primary mode of antitumor activity for Alimta has previously been ascribed to inhibition of thymidylate synthase (TS) (1). However, several lines of evidence strongly suggest that multiple enzyme-inhibitory mechanisms are involved in cytotoxicity: 1) the reversal pattern for Alimta in human breast and colon carcinoma cell lines demonstrate that, although TS may be a major mechanism for cytotoxic action at Alimta concentrations near the IC_{50}, higher concentrations can lead to inhibition of dihydrofolate reductase (DHFR) and/or other enzymes along the purine *de novo* pathway (2); 2) secondary mechanisms of cytotoxicity aside from TS modulation predominate in tumor cell populations that were raised for resistance to Alimta and the selective TS inhibitor, tomudex (3); 3) Alimta is an excellent substrate for folylpolyglutamate synthetase, and the Ki values of the pentaglutamate are 1.3, 7.2, and 65 nM for inhibition of TS, DHFR, and glycinamide ribonucleotide formyltransferase (GARFT), respectively (2); 4) intracellular concentrations of Alimta and its polyglutamates exceed 40 μM in human leukemia cells (4); and 5) early clinical studies demonstrated that patients who had previously failed to respond to raltitrexed and 5-fluorouracil/leucovorin treatment responded to Alimta (5; DA Rinaldi, personal communication).

Alimta is undergoing broad phase II evaluation as a single agent, and preliminary results show responses in several tumor types, including breast cancer (6-9). Doxorubicin is one of the most active chemotherapy drugs used in the treatment of breast cancer. Because Alimta and doxorubicin have mechanisms of action that might be complementary, we investigated a possible synergism between doxorubicin and Alimta on growth inhibition of ZR-75-1 human breast carcinoma cells.

Materials and Methods

Reagents. Doxorubicin hydrochloride, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT), triton X-100, and DNase-free ribonuclease A [R-6513] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propidium iodide was obtained from Molecular Probes (Eugene, OR, USA). Alimta was synthesized at Eli Lilly and Co. (1).

Cell culture. The ZR-75-1 human breast carcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD,

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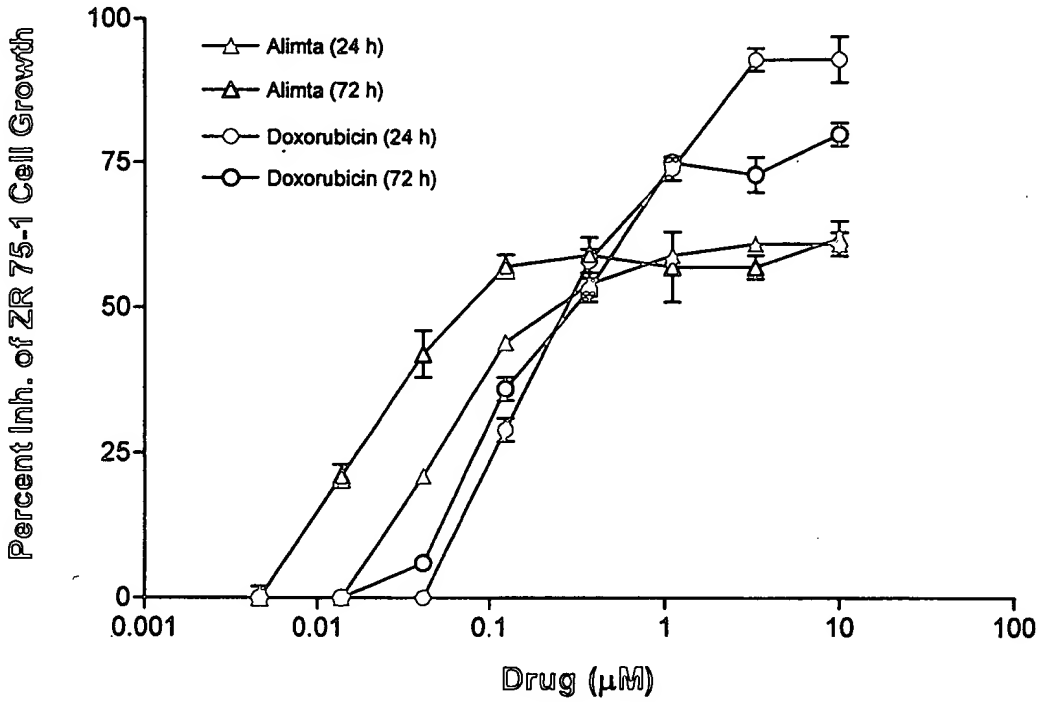

Figure 1. *Concentration response data for inhibition of ZR-75-1 cell growth by Alimta administered for 24 hours starting at the time of plating or for 72 hours starting 24 hours after plating for a total culture time of 96 hours. The M ± SE of triplicate determinations is shown.*

USA). It was originally derived from a malignant ascitic effusion in a 63-year-old Caucasian female with infiltrating ductal carcinoma (10). The cells were cultured in RPMI-1640 medium containing L-glutamine and 25 mM HEPES buffer (Whittaker Bioproducts, Walkersville, MD, USA) and supplemented with 10% dialyzed fetal calf serum (Hyclone Laboratories, Inc., Logan, UT, USA). The cells were tested and found free of mycoplasma contamination by the American Type Culture Collection.

In vitro cytotoxicity testing. We used a modification of the original MTT colorimetric assay described by Mosmann (11) to measure cell cytotoxicity. The ZR-75-1 cells were seeded at 5000 cells/180 μl culture medium per well in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA) 24 hours prior to the addition of test compounds. Well 1A was left blank (200 μl culture medium without cells). The cells were exposed to Alimta or doxorubicin as single agents and combinations for 24 hours starting at the time of plating or for 72 hours starting 24 hours after plating with a total culture time of 96 hours. The disodium salt of Alimta was prepared in RPMI-1640 medium at 200 μM, and a series of three-fold dilutions were subsequently made in culture medium. Doxorubicin hydrochloride was prepared in 0.83% saline at 200 μM, and a series of three-fold dilutions were subsequently made in 0.83% saline. The series of compound dilutions were added to triplicate wells containing ZR-75-1 cells at 10 μl per well. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2-in-air. MTT was dissolved in phosphate-buffered saline at 5 mg/ml. Following incubation of the plates, 10 μl of stock MTT solution was added to all the wells of an assay, and the plates were incubated at 37°C for one additional hour. Following incubation, 100 μl dimethyl sulfoxide was added to each well. Following thorough formazan solubilization, the plates were read on a spectraMAX250 reader (Molecular Devices Corp., Sunnyvale, CA, USA) using a test wavelength of 570 nm.

The drug combination data was analyzed using the CalcuSyn program for dose effect analysis (Biosoft, Cambridge, UK). The combination effect was determined to be synergistic for CI<1, additive for CI = 1, and antagonistic for CI>1 (12).

Flow cytometry assay. Log-phase ZR-75-1 human breast carcinoma cells were seeded at a density of 1 x 10^6 cells in T-75 flasks (Corning, Cambridge, MA, USA) containing 25 ml of RPMI-1640 media supplemented with 10% dialyzed fetal bovine serum and 25 mM HEPES buffer. The cells were incubated at 37°C for 24 hours prior to addition of the drug. A 2mM stock solution of Alimta was prepared in 0.83% saline and further diluted in RPMI-1640 medium. Alimta was added to ZR-75-1 cells at a concentration of 33 nM for incubation times of 6, 24 and 48 hours. After each incubation time, the cells were harvested, washed with Dulbecco's phosphate-buffered saline (PBS; BioWhittaker, Inc.) and then centrifuged. The cell pellet was resuspended in 0.5 ml of PBS. Cold 70% ethanol (4.5 ml) was added to the cell suspension and mixed. The cells were then stored at -20°C for 24 hours. After removing them from -20°C, the cells were centrifuged and washed once in PBS. One-ml of staining solution (10 ml of 0.1% Triton X-100 [Sigma] containing 2 mg DNase-free RNase A [Sigma] and 20 μg of propidium iodide [Molecular Probes, Eugene, OR]) was added to the cell pellet. The cells were analyzed on a Coulter Epics Flow Cytometer.

Results

Effect of exposure time on cytostatic activity of alimta and doxorubicin. We tested the effects of Alimta and doxorubicin exposure (24 or 72 hours) on the growth inhibitory activity toward ZR 75-1 cells. The cells were exposed to drug for 24

Table I. *Effect of Alimta pretreatment (24 hours) on inhibition of cell growth of ZR-75-1 human breast cancer cells by doxorubicin.*

Doxorubicin (μM)	% Growth Inhibition with Alimta (μM)[a]			
	0	0.041	0.123	0.37
0	1 ± 1[a]	3 ± 2	29 ± 3	54 ± 6
0.000508	0 ± 0	42 ± 3	SS ± 2	65 ± 2
0.00152	4±3	48±2	56±3	66±1
0.00457	3±3	43±3	S1±1	53±3
0.0137	1 ± 1	43 ± 3	57 ± 2	S9 ± 1
0.041	4±2	56±4	53 ±3	56±4
0.123	20±3	58±0	63 ± 1	55±2
0.37	32±3	58±2	62±2	61 ±3
1.11	53±3	75±2	83±1	90±1
3.33	65±4	85±3	76±1	84±2
10	63±0	83±2	72±0	80±1

[a]Percent growth inhibition determined by MTT analysis after 72-hour doxorubicin exposure
[b]M ± SEM obtained from triplicate determinations.

Table II. *Inhibitory effect of Alimta (0-24 hours pre-exposure) followed by doxorubicin (24-96 hours) treatment on ZR 75-1 cell growth.*

Alimta (μM)	Doxorubicin (μM)	Fraction affected CI	value[a]
0.041	0.000508	0.42	0.174
0.041	0.00152	0.48	0.151
0.041	0.00457	0.43	0.176
0.041	0.0137	0.43	0.189
0.041	0.041	0.56	o.lS1
0.123	0.000508	0.55	0.379
0.123	0.00152	0.56	0.371
0.123	0.00457	0.51	0.423
0.123	0.0137	0.57	0.372
0.123	0.041	0.53	0.429
0.370	0.000508	0.65	0.884
0.370	0.00152	0.66	0.862
0.370	0.00457	0.53	1.203
0.370	0.0137	0.59	1.047
0.370	0.041	0.56	1 138

[a]The data was analyzed for combination index (CI) values based on mutually non-exclusive case and actual fraction affected. CI values < 1, 1, and >1 indicate syngergism, additivity and antagonism, respectively.

hours starting at the time of plating or for 72 hours starting 24 hours after plating with a total culture time of 96 hours. The growth inhibitory IC_{50}s for Alimta or doxorubicin were 85 and 326 nM, respectively (72-hour exposures) and 271 and 302 nM, respectively (24-hour exposures). The concentration response data for these exposure periods are shown in Figure 1.

Alimta/doxorubicin combinations in ZR 75-1 cells. Representative data for Alimta pretreatment (24 hours) followed by doxorubicin is shown in Table I. This combination was extremely synergistic so that concentrations of each drug that lacked activity by themselves produced potent growth inhibition when administered sequentially. For example, Alimta alone at 0.041 μM (0 - 24 h; 3% growth inhibition) plus doxorubicin alone at 0.00152 μM (24 - 96 h; 4% growth inhibition) produced 48% growth inhibition in combination (Table I). The dose effect analysis using the CalcuSyn program is shown in Table II. Strong synergy was noted at sub IC_{50} concentrations of Alimta.

The dose effect analysis for simultaneous 72-hour exposures to Alimta plus doxorubicin is shown in Table III. With concentrations of Alimta below the IC_{50} (<85 nM; Figure 1), the response was primarily additive over a wide range of doxorubicin concentrations. However, Alimta at the higher concentration (370 nM) produced antagonistic results.

The dose effect analysis for doxorubicin pretreatment (0 - 24 hours) followed by Alimta (24 - 96 hours) is shown in Table IV. At the lowest Alimta concentration (13.7 nM), the combined effect ranged from strong antagonism to synergism with increasing doxorubicin concentration. At the intermediate Alimta concentration (41 nM), the combined effect ranged from synergistic to modest antagonism with increasing doxorubicin concentration. The highest Alimta concentration (123 nM) produced only antagonistic results.

Alimta-induced cell cycle effects. The cell cycle effects of Alimta were evaluated using flow cytometry. DNA content histograms of ZR-75-1 cells treated with Alimta at 33 nM for different time intervals are shown in Figure 2. At the 24 hour time-point, the cells began to accumulate at the G_1/S-interphase and, by 48 hours, most of these cells had accumulated at this interphase.

Table III. *Inhibitory effect of simultaneous 72-hour Alimta and doxorubicin (24-96 hours) treatment on ZR 75-1 cell growth.*

Alimta (μM)	Doxorubicin (μM)	Fraction affected CI	value[a]
0.041	0.0046	0.38	1.07
0.041	0.0137	0.30	2.60
0.041	0.041	0.36	1.36
0.041	0.123	0.40	0.97
0.041	0.37	0.38	1.41
0.041	1.11	0.61	0.45
0.123	0.0046	0.39	2.90
0.123	0.0137	0.38	3.22
0.123	0.041	0.49	1.09
0.123	0.123	0.45	1.66
0.123	0.37	0.46	1.66
0.123	1.11	0.56	0.97
0.370	0.0046	0.53	2.16
0.370	0.0137	0.47	3.90
0.370	0.041	0.51	2.65
0.370	0.123	0.47	3.97
0.370	0.37	0.49	3.40
0.370	1.11	0.57	1.87

[a]The data were analyzed for combination index (CI) values based on mutually non-exclusive case and actual fraction affected. CI values < 1, 1, and >1 indicate syngergism, additivity and antagonism, respectively.

Table IV. *Inhibitory effect of doxorubicin (0-24 hours pre-exposure) followed by Alimta (24-96 hours) treatment on ZR 75-1 cell growth.*

Doxorubicin (μM)	Alimta (μM)	Fraction affected CI	value[a]
0.0046	0.0137	0.00	6.75
0.0137	0.0137	0.00	7.58
0.041	0.0137	0.00	10.06
0.123	0.0137	0.42	1.30
0.37	0.0137	0.52	0.49
1.11	0.0137	0.70	0.68
0.0046	0.041	0.49	0.58
0.0137	0.041	0.44	0.64
0.041	0.041	0.45	1.08
0.123	0.041	0.42	1.30
0.37	0.041	0.52	1.03
1.11	0.041	0.68	1.60
0.0046	0.123	0.50	1.71
0.0137	0.123	0.52	1.67
0.041	0.123	0.42	1.77
0.123	0.123	0.42	2.04
0.37	0.123	0.56	1.40
1.11	0.123	0.67	1.46

[a]The data were analyzed for combination index (CI) values based on mutually non-exclusive case and actual fraction affected. CI values < 1, 1, and >1 indicate syngergism, additivity and antagonism, respectively.

Discussion

Alimta is undergoing broad phase II evaluation as a single agent and preliminary results show responses in several tumor types, including NSCLC, breast, colorectal and mesothelioma (6-9, 13). An important component in the development of a new anticancer drug is an understanding of its potential for inclusion in combination treatment regimens. Teicher *et al.* demonstrated that the combination of Alimta and doxorubicin produced additive tumor growth delay in the NCI H460 non-small cell lung carcinoma (14). In the present communication, we show that the interaction of Alimta and doxorubicin is highly sequence dependent. Preincubation with Alimta for 24 hours followed by exposure to doxorubicin for 72 hours resulted in highly synergistic activity, whereas the opposite sequence produced additive or antagonistic responses.

The mechanism by which Alimta pretreatment produces synergistic activity with doxorubicin in ZR-75-1 breast carcinoma cells is not presently understood. DNA flow cytometry studies indicate that MTA causes a buildup of cells near the G_1/S interface after 24 hours of incubation. Doxorubicin has been shown to intercalate between adjoining nucleotide pairs in the DNA helix structure (15) and induce protein-linked double-strand DNA breaks. It is possible that Alimta causes the synchronization of the population in early S-phase following release of the block where doxorubicin is maximally cytotoxic(16), although

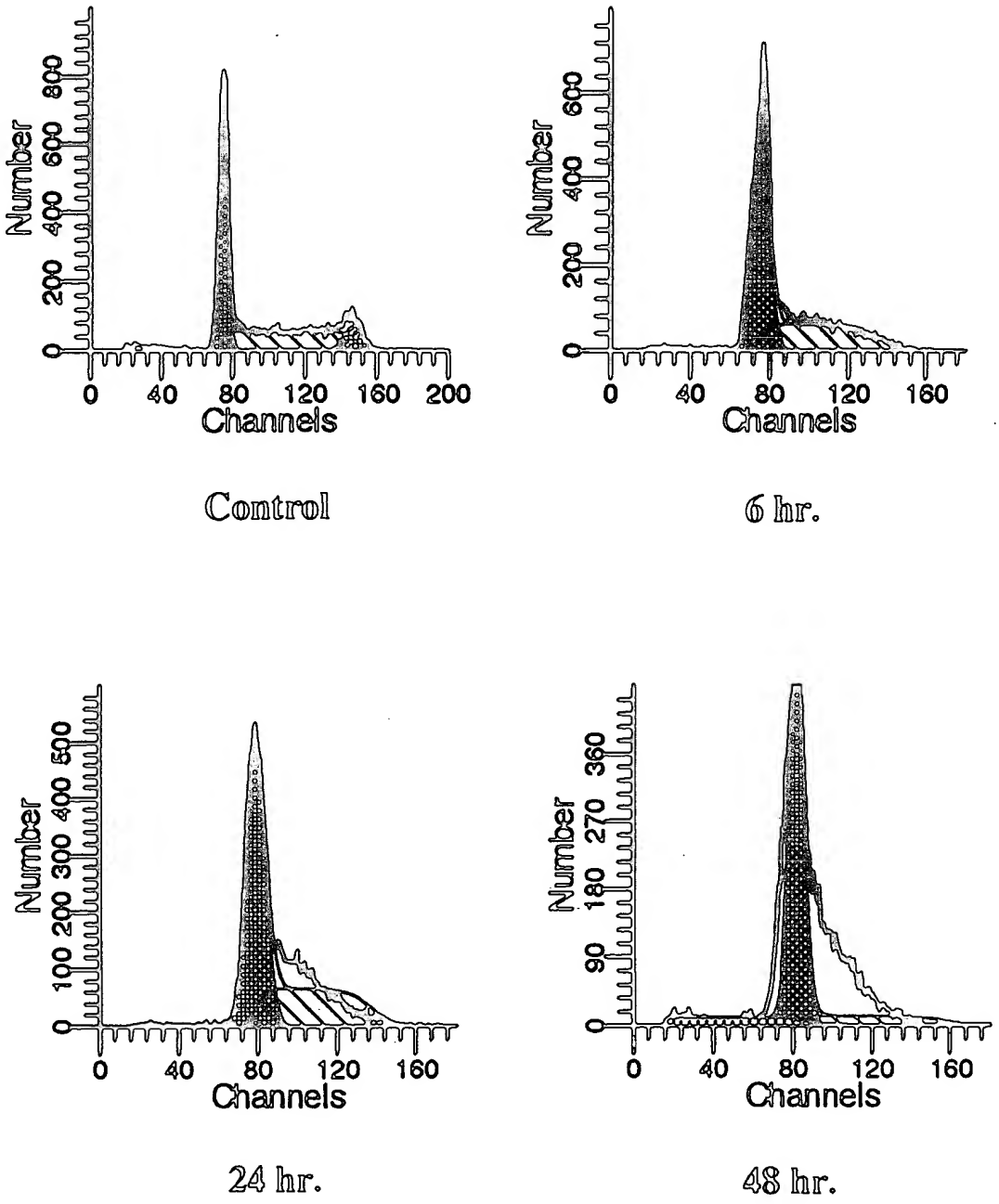

Figure 2. *DNA content histograms of ZR-75-1 human breast carcinoma cells treated with 33 nM Alimta. The data from a representative experiment with different intervals of treatment are shown.*

doxorubicin appears to be active in all phases of the cell cycle. Similarly, antagonistic interactions are most evident when the cells are exposed to the two drugs simultaneously: Doxorubicin is most active on cycling cells and Alimta appears to block the cells at the G_1/S interface. From these studies it is obvious that the mechanism of potentiation was dependent on temporally spaced events. Additional *in vitro* studies are needed to further elucidate the mechanisms of potentiation of cytotoxicity by this drug combination. The studies to date suggest that Alimta should precede doxorubicin when the drugs are given in combination chemotherapy protocols.

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